(54) Title: MUTANT ENTEROTOXIN EFFECTIVE AS A NON-TOXIC ADJUVANT FOR HIV

(57) Abstract

Methods and compositions are provided herein for the use of a novel mutant form of E. coli heat-labile enterotoxin which has lost its toxicity but has retained its immunologic activity. This enterotoxin is used in combination with an unrelated antigen to achieve an increased immune response to said antigen when administered as part of a vaccine preparation.

<u>MUTANT ENTEROTOXIN EFFECTIVE AS A NON-TOXIC ADJUVANT FOR HIV</u>

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5 invention.

1.   <u>FIELD OF THE INVENTION</u>

The present invention is directed towards a genetically distinct mutant of <u>E</u>. <u>coli</u> heat-labile
10 enterotoxin (LT) and its use as an adjuvant to induce mucosal and serum antibodies and cell mediated immune responses. Specifically, the mutant LT is modified by a single amino acid substitution that reduces its inherent toxicity but leaves intact the adjuvant properties of the molecule.

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2.   <u>BACKGROUND OF THE INVENTION</u>

Microbial pathogens can infect a host by one of several mechanisms. They may enter through a break in the integument induced by trauma, they may be introduced by
20 vector transmission, or they may interact with a mucosal surface. The majority of human pathogens initiate disease by the last mechanism, i.e., following interaction with mucosal surfaces. Bacterial and viral pathogens that act through this mechanism first make contact with the mucosal surface where
25 they may attach and then colonize, or be taken up by specialized absorptive cells (M cells) in the epithelium that overlay Peyer's patches and other lymphoid follicles [Bockman and Cooper, 1973, Am. J. Anat. 136:455-477; Owen et al., 1986, J. Infect. Dis. 153:1108-1118]. Organisms that enter
30 the lymphoid tissues may be readily killed within the lymphoid follicles, thereby provoking a potentially protective immunological response as antigens are delivered to immune cells within the follicles (e.g., <u>Vibrio</u> <u>cholerae</u>). Alternatively, pathogenic organisms capable of surviving
35 local defense mechanisms may spread from the follicles and subsequently cause local or systemic disease (i.e.,

Salmonella spp., poliovirus, rotavirus in immunocompromised
hosts).

Secretory IgA (sIgA) antibodies directed against
specific virulence determinants of infecting organisms play
5 an important role in overall mucosal immunity [Cebra et al.,
1986, In: Vaccines 86, Brown et al. (ed.), Cold Spring Harbor
Laboratory, New York. p.p. 129-133]. In many cases, it is
possible to prevent the initial infection of mucosal surfaces
by stimulating production of mucosal sIgA levels directed
10 against relevant virulence determinants of an infecting
organism. Secretory IgA may prevent the initial interaction
of the pathogen with the mucosal surface by blocking
attachment and/or colonization, neutralizing surface acting
toxins, or preventing invasion of the host cells. While
15 extensive research has been conducted to determine the role
of cell mediated immunity and serum antibody in protection
against infectious agents, less is known about the
regulation, induction, and secretion of sIgA. Parenterally
administered inactivated whole-cell and whole-virus
20 preparations are effective at eliciting protective serum IgG
and delayed type hypersensitivity reactions against organisms
that have a significant serum phase in their pathogenesis
(i.e., _Salmonella_ _typhi_, Hepatitis B). However, parenteral
vaccines are not effective at eliciting mucosal sIgA
25 responses and are ineffective against bacteria that interact
with mucosal surfaces and do not invade (e.g., _Vibrio_
cholerae). There is, however, recent evidence that
parenterally administered vaccines may be effective against
at least one virus, rotavirus, that interacts primarily with
30 mucosal surfaces [Conner et al., 1993, J. Virol.
67:6633-6641]. Protection is presumed to result from
transudation of antigen specific IgG onto mucosal surfaces
for virus neutralization. Therefore, mechanisms that
stimulate both serum and mucosal antibodies and cell mediated
35 immunity are important for effective vaccines.

Mucosal immunization can be effective for induction
of specific sIgA responses if the antigens are presented to

the T and B lymphocytes and accessory cells contained within
the Peyer's patches where preferential IgA B-cell development
is initiated. The Peyer's patches contain helper T (TH)-cells
that mediate B-cell isotype switching directly from IgM cells
5 to IgA B-cells. The patches also contain T-cells that
initiate terminal B-cell differentiation. The primed B-cells
then migrate to the mesenteric lymph nodes and undergo
differentiation, enter the thoracic duct, then the general
circulation, and subsequently seed all of the secretory
10 tissues of the body, including the lamina propria of the gut
and respiratory tract. IgA is then produced by the mature
plasma cells, complexed with membrane-bound Secretory
Component, and transported onto the mucosal surface where it
is available to interact with invading pathogens [Strober and
15 Jacobs, 1985, In: Advances in host defense mechanisms. Vol.
4. Mucosal Immunity, Gallin and Fauci (ed.), Raven Press, New
York. p.p. 1-30; Tomasi and Plaut, 1985, In: Advances in host
defense mechanisms. Vol. 4. Mucosal Immunity, Gallin and
Fauci (ed.), Raven Press, New York. p.p. 31-61]. The
20 existence of this common mucosal immune system explains in
part the potential of live attenuated vaccines and mucosal
immunization for protection against pathogenic organisms that
initiate infection by first interacting with mucosal
surfaces.
25   A number of strategies have been developed for
mucosal immunization, including the use of attenuated mutants
of bacteria (i.e., Salmonella spp.) as carriers of
heterologous antigens [Cárdenas and Clements, 1992, Clin.
Microbiol. Rev. 5:328-342; Clements et al., 1992, In:
30 Recombinant DNA Vaccines: Rationale and Strategy, Isaacson
(ed.), Marcel Decker, New York. p.p. 293-321; Clements and
Cárdenas, 1990, Res. Microbiol. 141:981-993; Clements and
El-Morshidy, 1984, Infect. Immun. 46:564-569], encapsulation
of antigens into microspheres composed of
35 poly-DL-lactide-glycolide (PGL), protein-like polymers -
proteinoids [Santiago et al., 1993, Pharmaceutical Research
10:1243-1247], gelatin capsules, different formulations of

liposomes [Alving et al., 1986, Vaccine 4:166-172; Garcon and
Six, 1993, J. Immunol. 146:3697-3702; Gould-Fogerite and
Mannino, 1993, In: Liposome Technology 2nd Edition. Vol. III,
Gregoriadis (ed.)], adsorption onto nanoparticles, use of

5 lipophilic immune stimulating complexes (ISCOMS) [Mowat and
Donachie, 1991, Immunology Today 12:383-385], and addition of
bacterial products with known adjuvant properties [Clements
et al., 1988, Vaccine 6:269-277; Elson, 1989, Immunology
Today 146:29-33; Lycke and Holmgren, 1986, Immunology

10 59:301-308; Lycke et al., 1992, Eur. J. Immunol. 22:2277-
2281]. The two bacterial products with the greatest potential
to function as mucosal adjuvants are cholera toxin (CT),
produced by various strains of V. cholerae, and the
heat-labile enterotoxin (LT) produced by some enterotoxigenic

15 strains of Escherichia coli. Although LT and CT have many
features in common, these are clearly distinct molecules with
biochemical and immunologic differences which make them
unique.

The extensive diarrhea of cholera is the result of
20 a potent exo-enterotoxin which causes the activation of
adenylate cyclase and a subsequent increase in intracellular
levels of cyclic $3'-,5'$-adenosine monophosphate (cAMP). The
cholera enterotoxin (CT) is an 84,000 dalton polymeric
protein composed of two major, non-covalently associated,

25 immunologically distinct regions or domains ("cholera-A" and
"cholera-B") [Finkelstein and LoSpalluto, 1969, J. Exp. Med.
130: 185-202]. Of these, the 56,000 dalton region, or
choleragenoid, is responsible for binding of the toxin to the
host cell membrane receptor, G_{MI} (galactosyl-

30 N-acetylgalactosaminyl- (sialyl)- galactosyl-glucosyl
ceramide), which is found on the surface of essentially all
eukaryotic cells. Choleragenoid is composed of five
non-covalently associated monomers, while the A region
(27,000 daltons) is responsible for the diverse biological

35 effects of the toxin.

The relationship of the two subunits of CT with
respect to the immunologic properties of the molecule has

been a source of considerable debate. On the one hand, CT is
an excellent immunogen that provokes the development of both
serum and mucosal antitoxin antibody responses when delivered
mucosally. This finding is not new in that cholera patients
5 are known to develop rises in titers of antitoxin antibodies
during convalescence from clinical cholera [Finkelstein,
1975, Curr. Top. Microbiol. Immunol. 69: 137-196]. One key
finding of those investigating the nature of this response
was the observation that CT, unlike most other protein
10 antigens, is not subject to the phenomenon known as oral
tolerance [Elson and Ealding, 1984, J. Immunol. 133:
2892-2897; Elson and Ealding, 1984, J. Immunol. 132:
2736-2741]. This was also found to be true when just the
B-subunit was fed to mice, an observation substantiated by
15 the cholera vaccine field trials in Bangladesh in which oral
immunization with B-subunit combined with killed whole cells
gave rise to mucosal as well as systemic antitoxin antibody
responses [Svennerholm et al., 1984, J. Infect. Dis. 149:
884-893].

20   In addition to being a potent mucosal immunogen, CT
has a number of other reported immunologic properties. As
indicated above, Elson and Ealding [Elson and Ealding, 1984,
J. Immunol. 133: 2892-2897] observed that orally administered
CT is not subject to oral tolerance. Moreover, simultaneous
25 oral administration of CT with a soluble protein antigen,
keyhole limpet hemocyanin (KLH), resulted in the development
of secretory IgA responses against both CT and KLH and also
abrogated the induction of oral tolerance against KLH. These
findings were subsequently confirmed and extended by Lycke
30 and Holmgren [Lycke and Holmgren, 1986, Immunology
59:301-308]. The confusion arises when one attempts to define
the role of the A and B subunits of CT with respect to the
adjuvant properties of the molecule. The following
observations, as summarized by Elson [Elson, 1989, Immunology
35 Today 146:29-33], are the basis for that confusion:

• CT is not subject to oral tolerance [Elson and Ealding,
1984, J. Immunol. 133: 2892-2897].

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- CT-B is not subject to oral tolerance [Elson and Ealding, 1984, J. Immunol. 133: 2892-2897].
- CT can prevent the induction of tolerance against other antigens with which it is simultaneously delivered and also

5 serve as an adjuvant for those antigens [Elson and Ealding, 1984, J. Immunol. 133: 2892-2897; Lycke and Holmgren, 1986, Immunology 59:301-308].

- CT can act as an adjuvant for CT-B [Elson and Ealding, 1984, J. Immunol. 133: 2892-2897].

10 • Heat aggregated CT has little toxicity but is a potent oral immunogen [Pierce et al., 1983, Infect. Immun. 40: 1112-1118].

- CT-B can serve as an immunologic "carrier" in a traditional hapten-carrier configuration [Cebra et al., 1986, In:

15 Vaccines 86, Brown et al. (ed.), Cold Spring Harbor Laboratory, New York. p.p. 129-133; McKenzie and Halsey, 1984, J. Immunol. 133: 1818-1824].

A number of researchers have concluded from these findings that the B-subunit must possess some inherent

20 adjuvant activity. The findings of Cebra et al. [Cebra et al., 1986, In: Vaccines 86, Brown et al. (ed.), Cold Spring Harbor Laboratory, New York. p.p. 129-133], Lycke and Holmgren [Lycke and Holmgren, 1986, Immunology 59:301-308], and Liang et al. [Liang et al., 1988, J. Immunol. 141: 1495-1501] would argue

25 against that conclusion. Cebra et al. [Cebra et al., 1986, In: Vaccines 86, Brown et al. (ed.), Cold Spring Harbor Laboratory, New York. p.p. 129-133] demonstrated that purified CT-B was effective at raising the frequency of specific anti-cholera toxin B-cells in Peyer's patches when given

30 intraduodenally but, in contrast to CT, did not result in significant numbers of IgA committed B-cells. Lycke and Holmgren [Lycke and Holmgren, 1986, Immunology 59:301-308] compared CT and CT-B for the ability to enhance the gut mucosal immune response to KLH by measuring immunoglobulin

35 secreting cells in the lamina propria of orally immunized mice. They found no increase in anti-KLH producing cells in response to any dose of B-subunit tested in their system.

Finally, Liang et al. [Liang et al., 1988, J. Immunol. 141:
1495-1501] found no adjuvant effect when CT-B was administered
orally in conjunction with inactivated Sendai virus.

      Where adjuvant activity has been observed for isolated
5 B-subunit, it has typically been for one of two reasons.
First, a traditional method of preparing B-subunit has been to
subject holotoxin to dissociation chromatography by gel
filtration in the presence of a dissociating agent (i.e.,
guanidine HCl or formic acid). The isolated subunits are then
10 pooled and the dissociating agent removed. B-subunit prepared
by this technique is invariably contaminated with trace
amounts of A-subunit such that upon renaturation a small
amount of holotoxin is reconstituted. The second reason has to
do with the definition of an immunologic carrier. Like many
15 other soluble proteins, B-subunit can serve as an immunologic
vehicle for presentation of antigens to the immune system. If
those antigens are sufficiently small as to be poorly
immunogenic, they can be made immunogenic in a traditional
hapten-carrier configuration. Likewise, there is a
20 "theoretical" immune enhancement associated with B-subunit,
especially for oral presentation, in that B-subunit binds to
the surface of epithelial cells and may immobilize an attached
antigen for processing by the gut associated lymphoid tissues.
However, any potential advantage to this mechanism of antigen
25 stabilization may be offset by the distribution of the antigen
across non-immunologically relevant tissues, i.e., the surface
of mucosal epithelial cells. In context of the mucosal
responsiveness, the immunologically relevant sites for
initiation of an immune response are the Peyer's patches,
30 especially for antigen-specific T cell-dependent B cell
activation [Strober and Jacobs, 1985, In: Advances in host
defense mechanisms. Vol. 4. Mucosal Immunity, Gallin and Fauci
(ed.), Raven Press, New York. p.p. 1-30; Tomasi and Plaut,
1985, In: Advances in host defense mechanisms. Vol. 4. Mucosal
35 Immunity, Gallin and Fauci (ed.), Raven Press, New York. p.p.
31-61; Brandtzaeg, 1989, Curr. Top. Microbiol. Immunol. 146:
13-25]. Thus, the events up to isotype switching from IgM

cells to IgA B-cells occurs in the Peyer's patches. Antigens
localized on the epithelial cell surface may contribute to
antigen induced B cell proliferation in that the class II
positive epithelial cells may act as antigen presenting cells
5 for T cell activation at the secretory site, thereby
increasing cytokine production, terminal B cell
differentiation, increased expression of secretory component,
and increased external transport of antigen specific IgA
[Tomasi, T. B., and A.G. Plaut. 1985, In: Advances in host
10 defense mechanisms. Vol. 4. Mucosal Immunity, Gallin and Fauci
(ed.), Raven Press, New York. p.p. 31-61]. The relationships
of these events have not been clearly defined for B-subunit as
a carrier of other antigens and use of the term "adjuvant"
would seem inappropriate for such an effect.

15   It is clear that the adjuvant property of the molecule
resides in the holotoxin in which B-subunit is required for
receptor recognition and to facilitate penetration of the
A-subunit into the cell. The A-subunit is also required for
adjuvant activity, presumably as a function of its
20 ADP-ribosylating enzymatic activity and ability to increase
intracellular levels of cAMP (see below). The B-subunit alone
may act as a carrier of other antigens in that when conjugated
to those antigens they can be immobilized for processing by
the mucosal associated lymphoid tissues.

25   Although LT and CT have many features in common, these
are clearly distinct molecules with biochemical and
immunologic differences which make them unique, including a
20% difference in nucleotide and amino acid sequence homology
[Dallas and Falkow, 1980, Nature 288: 499-501]. The two toxins
30 have the same subunit number and arrangement, same biological
mechanism of action, and the same specific activity in many in
vitro assays [Clements and Finkelstein, 1979, Infect. Immun.
24:760-769; Clements et al., 1980, Infect. Immun. 24: 91-97].

     There are, however, significant differences between
35 these molecules that influence not only their enterotoxic
properties, but also their ability to function as adjuvants.
To begin with, unlike CT produced by V. cholerae, LT remains

cell associated and is only released from E. coli during cell
lysis [Clements and Finkelstein, 1979, Infect. Immun.
24:760-769]. CT is secreted from the vibrio as soon as it is
synthesized and can be readily identified in, and purified
5 from, culture supernatants. Consequently, in contrast to CT,
LT is not fully biologically active when first isolated from
the cell. Consistent with the A-B model for bacterial toxins,
LT requires proteolysis and disulfide reduction to be fully
active. In the absence of proteolytic processing, the
10 enzymatically active A_1 moiety is unable to dissociate from
the A_2 component and cannot reach its target substrate
(adenylate cyclase) on the basolateral surface of the
intestinal epithelial cell. This is also true for CT, but
proteases in the culture supernatant, to which the toxin is
15 exposed during purification, perform the proteolysis. Since LT
is not fully biologically active, it is difficult to identify
during purification using *in vitro* biological assays such as
the Y-1 adrenal cell assay or permeability factor assay.

        The result of this difference in biological activity
20 between LT and CT can be demonstrated as a difference in
threshold toxicity between these molecules. For example, in
in vitro tests such as cultured Y-1 mouse adrenal tumor cells,
there is an approximately 3-log difference in the biological
activity of un-nicked LT and CT. *In vivo*, CT induces
25 detectable net fluid secretion in the rabbit ligated ileal
loops at a dose of 0.1 μg while LT induces net secretion in
this same model at a dose of 5 μg. Significantly, when LT is
exposed to proteolytic enzymes with trypsin-like specificity,
the molecule becomes indistinguishable from CT in any biologic
30 assay system. This was demonstrated clearly by Clements and
Finkelstein [Clements and Finkelstein, 1979, Infect. Immun.
24:760-769].

        In addition to the above reported differences, LT has
an unusual affinity for carbohydrate containing matrices.
35 Specifically, LT, with a molecular weight of 90,000, elutes
from Sephadex columns (glucose) with an apparent molecular
weight of 45,000 and from Agarose columns (galactose) with an

apparent molecular weight of O. That is, it binds to galactose
containing matrices and can be eluted from those matrices in
pure form by application of galactose. LT binds not only to
agarose in columns used for purification, but more
5 importantly, to other biological molecules containing
galactose, including glycoproteins and lipopolysaccharides.
This lectin-like binding property of LT results in a broader
receptor distribution on mammalian cells for LT than for CT
which binds only to G_{MI}. This may account in part for the
10 reported differences in the abilities of these two molecules
to induce different helper T lymphocyte responses [McGhee et
al., 1994, Mucosal Immunology Update, Spring 1994, Raven
Press, New York. p. 21].

In these studies reported by McGhee et al. [McGhee et
15 al., 1994, Mucosal Immunology Update, Spring 1994, Raven
Press, New York. p. 21], it was shown that oral immunization
of mice with vaccines such as tetanus toxoid (TT) with CT as a
mucosal adjuvant selectively induces T_H2 type cells in Peyer's
patches and spleens as manifested by TH cells which produce
20 IL-4 and IL-5, but not IL-2 or INF-gamma. [For a more complete
review of the cytokine network see Arai et al., 1990, Ann.
Rev. Biochem. 59:783-836] <u>Importantly, when CT was used as a
mucosal adjuvant it also enhanced antigen-specific IgE
responses in addition to the IgA response.</u> Such enhancement of
25 IgE responses seriously compromises the safety of CT as a
mucosal adjuvant due to the prospect of inducing
immediate-type hypersensitivity reactions. In contrast, LT
induces both T_H1 and T_H2 cells and predominantly
antigen-specific IgA responses without IgE responses when used
30 as an orally administered mucosal adjuvant.

The two molecules also have many immunologic
differences, as demonstrated by immunodiffusion studies
[Clements and Finkelstein, 1978, Infect. Immun. 21: 1036-1039;
Clements and Finkelstein, 1978, Infect. Immun. 22: 709-713],
35 *in vitro* neutralization studies, and the partial protection
against LT associated <u>E</u>. <u>coli</u> diarrhea in volunteers receiving

B-subunit whole cell cholera vaccine [Clemens et al., 1988, J.
Infect. Dis. 158: 372-377].

Taken together, these findings demonstrate that LT and
CT are unique molecules, despite their apparent similarities,
5 and that LT is a practical mucosal adjuvant while CT is not.

The demonstration of the adjuvant properties of LT
grew out of an investigation of the influence of LT on the
development of tolerance to orally administered antigens by
one of the present inventors. It was not clear whether or not
10 LT would also influence the induction of oral tolerance or
exhibit the adjuvant effects demonstrated for CT, given the
observed differences between the two molecules. Consequently,
the present inventors examined a number of parameters,
including the effect of LT on oral tolerance to ovalbumin
15 (OVA) and the role of the two subunits of LT in the observed
response, the effect of varying the timing and route of
delivery of LT, the effect of prior exposure to OVA on the
ability of LT to influence tolerance to OVA, the use of LT as
an adjuvant with two unrelated antigens, and the effect of
20 route of immunization on anti-OVA responses. The results
obtained from these studies [Clemens et al., 1988, Vaccine
6:269-277; Clemens et al., 1988, Abstract No. B91, 88th Ann.
Meet. Am. Soc. Microbiol.] are summarized below:

1. Simultaneous administration of LT with OVA was shown to
25   prevent the induction of tolerance to OVA and to increase
      the serum anti-OVA IgG response 30 to 90 fold over OVA
      primed and PBS primed animals, respectively. This effect
      was determined to be a function of the enzymatically active
      A-subunit of the toxin since the B-subunit alone was unable
30   to influence tolerance induction.

2. Animals fed LT with OVA after an initial OVA prime developed
      a significantly lower serum IgG and mucosal IgA anti-OVA
      response than those fed LT with OVA in the initial
      immunization, indicating that prior exposure to the antigen
35   reduces the effectiveness of LT to influence tolerance and
      its ability to act as an adjuvant. LT was not able to
      abrogate tolerance once it had been established. This was

also found to be true for CT when animals were
pre-immunized with OVA prior to oral ovalbumin plus CT and
offers some insight into the beneficial observation that
antibody responses to nontarget dietary antigens are not
5   increased when these adjuvants are used.

3. Serum IgG and mucosal IgA responses in animals receiving LT
on only a single occasion, that being upon first exposure
to antigen, were equivalent to responses after three OVA/LT
primes, indicating that commitment to responsiveness occurs
10   early and upon first exposure to antigen. It was also
demonstrated that the direction of the response to either
predominantly serum IgG or mucosal IgA can be controlled by
whether or not a parenteral booster dose is administered.

4. Simultaneous administration of LT with two soluble protein
15   antigens results in development of serum and mucosal
antibodies against both antigens if the animal has no prior
immunologic experience with either. This was an important
finding since one possible application of LT as an adjuvant
would be for the development of mucosal antibodies against
20   complex antigens, such as killed bacteria or viruses, where
the ability to respond to multiple antigens would be
important.

Studies by Tamura et al., [Tamura et al., U.S. Patent
No. 5,182,109] demonstrated that LT and/or CT administered
25 intranasally (i.n.) enhanced the antibody titer against a co-
administered antigen. However, nowhere in Tamura et al. is it
taught that these toxins can induce a protective immune
response when administered orally.

Clearly, LT has significant immunoregulatory
30 potential, both as a means of preventing the induction of
tolerance to specific antigens and as an adjuvant for
mucosally administered antigens and it elicits the production
of both serum IgG and mucosal IgA against antigens with which
it is delivered. This raises the possibility of an effective
35 immunization program against a variety of pathogens involving
the mucosal administration of killed or attenuated agents or
relevant virulence determinants of specific agents. However,

the fact that this "toxin" can stimulate a net lumenal
secretory response when proteolytically cleaved, as by gut
proteases, or when administered in high enough concentrations
orally, may hinder investigation into its potential or prevent
5 its use under appropriate conditions. This problem could be
resolved if LT could be "detoxified" without diminishing the
adjuvant properties of the molecules. In order to appreciate
how this might be accomplished, it is necessary to further
analyze the mechanism of action of the LT and CT and the
10 structural and functional relationships of these molecules. As
indicated previously, both LT and CT are synthesized as
multisubunit toxins with A and B components. After the initial
interaction of the toxin with the host cell membrane receptor,
the B region facilitates the penetration of the A-subunit
15 through the cell membrane. On thiol reduction, this A
component dissociates into two smaller polypeptide chains. One
of these, the A_1 piece, catalyzes the ADP-ribosylation of the
stimulatory GTP-binding protein (G_s) in the adenylate cyclase
enzyme complex on the basolateral surface of the epithelial
20 cell and this results in increasing intracellular levels of
cAMP. The resulting increase in cAMP causes secretion of water
and electrolytes into the small intestine through interaction
with two cAMP-sensitive ion transport mechanisms involving 1)
NaCl co-transport across the brush border of villous
25 epithelial cells, and 2) electrogenic Na^+ dependent Cl^-
secretion by crypt cells [Field, 1980, In: Secretory diarrhea,
Field et al. (ed.), Waverly Press, Baltimore. p.21-30]. The A
subunit is also the principal moiety associated with immune
enhancement by these toxins. This subunit then becomes a
30 likely target for manipulation in order to dissociate the
toxic and immunologic functions of the molecules. A recent
report by Lycke et al. [Lycke et al., 1992, Eur. J. Immunol.
22:2277-2281] makes it clear that alterations that affect the
ADP-ribosylating enzymatic activity of the toxin and alter the
35 ability to increase intracellular levels of cAMP also prevent
the molecule from functioning as an adjuvant. Consequently,
another approach to detoxification must be explored.

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3.   SUMMARY OF THE INVENTION

The present invention is based on the surprising observation that a mutant form of LT, which has reduced toxicity and is devoid of *in vitro* ADP-ribosyltransferase activity, still retains its activity as an immunological adjuvant when administered, preferably through a mucosal surface, e.g. orally, nasally, etc. The mutant form of LT differs from the wild-type by a single amino acid substitution, Arg_{192}-Gly_{192}, rendering a trypsin sensitive site insensitive. The loss of the proteolytic site prevents the proteolytic processing of the A subunit into its toxic form. Native LT is not toxic when first isolated from the bacterium but has the potential to be fully toxic when exposed to proteases such as those found in the mammalian intestine. The mutant form of LT no longer has the potential to become toxic due to proteolytic activation. This mutant LT (hereinafter mLT) retains the capability of enhancing an animal's immune response (e.g., IgG, IgA, cell mediated immunity) to an antigen unrelated to LT or mLT with no toxic side effects at an adjuvant effective dose. Experimental evidence shows that mLT has utility as an adjuvant for administered antigens; such administration results in the production of serum IgG and/or mucosal sIgA and cell mediated immune responses against the antigen with which the mLT is delivered.

The present invention provides a method for induction of a serum and/or mucosal immune response in a host to any antigen administered, by way of example and not by way of limitation, by an intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, transdermal, epidural, pulmonary, oral, nasal, gastric, intestinal, rectal, vaginal, or urethral route. Preferably, the route of administration is a mucosal route of administration, i.e., through a mucosal membrane or surface, such as an oral, nasal, gastric, intestinal, rectal, vaginal or urethral route. More preferably, the mucosal route of administration is through an oral or nasal membrane.

The methods of the present invention comprise administering to the host an effective amount of mLT in

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conjunction with administration of an effective amount of the
antigen. Preferably, the antigen and the mLT are administered
initially in a simultaneous dose.

The present method and compositions provide an
5 improved mode of immunization for development of serum and
mucosal antibodies and cell mediated immune responses against
pathogenic microorganisms. Production of IgA antibody
responses against pathogenic microorganisms which penetrate or
invade across mucosal surfaces can be directed to that
10 surface, while a significant serum antibody and cell mediated
immune responses can be developed to prevent infection by
pathogenic microorganisms against which serum antibody or cell
mediated immunity is protective. The present invention is
useful for any specific antigen where a specific neutralizing
15 antibody or cell mediated immune response would be useful in
ablating the physiological or disease state associated with
that antigen.

The present invention also provides a composition
useful as a component of a vaccine against enterotoxic
20 bacterial organisms expressing cholera-like enterotoxins and
methods for its use.

The invention also provides a composition useful in
these methods. The composition comprises an effective amount
of mLT in combination with an effective amount of antigen.

25

4. BRIEF DESCRIPTION OF THE FIGURES

The present invention may be understood more fully by
reference to the following detailed description of the
invention, examples of specific embodiments of the invention
30 and the appended figures in which:

Figure 1 is a schematic diagram of the plasmid pBD94,
which encodes both subunits A and B under the control of the
lac promoter. Plasmid pBD95 contains the single base
substitution at amino acid residue 192 of subunit A, coding
35 for Gly rather than Arg, which preserves the reading frame but
eliminates the proteolytic site. The amino acid sequence

corresponding to the region of trypsin sensitivity and the site of the amino acid substitution $Arg_{192}-Gly_{192}$ is shown.

Figure 2 is a graphic demonstration of the dose-dependent increase in the levels of ADP-ribosylagmatine as a function of increasing amounts of CT.

Figure 3 shows the fluid accumulation after feeding 125 μg of native LT but not after feeding 125 μg of mLT to mice. The gut-carcass ratio is defined as the intestinal weight divided by the remaining carcass weight.

Figure 4 illustrates the ability of mLT to act as an immunological adjuvant. Figure 4A shows the ability of mLT to induce a serum IgG response to OVA. Figure 4B shows the ability of mLT to induce a mucosal sIgA response to OVA.

Figure 5 is an experimental demonstration that mLT retains the ability to prevent induction of oral tolerance to LT. Figure 5A shows the ability of mLT to induce a serum IgG response to LT. Figure 5B shows the ability of mLT to induce a mucosal sIgA response to LT.

Figure 6 illustrates anti-HIV serum IgG levels and nasal and vaginal IgA levels of mice immunized intranasally (i.n.) with HIV-1 viral lysate (HIV), either with or without mLT (LT(R192G)). See text of Section 7.1 for details.

Figure 7 illustrates anti-gp120 serum IgG levels of mice immunized intranasally (i.n.) or subcutaneously (s.c.) with gp120 with or without mLT (LT(R192G)), and with or without an intraperitoneal (i.p.) boost. See text of Section 7.2 for details.

Figure 8 illustrates anti-gp120 vaginal IgA levels of mice immunized intranasally (i.n.) or subcutaneously (s.c.) with gp120 with or without mLT (LT(R192G)), and with or without an i.p. boost. See text of Section 7.2 for details.

Figure 9 shows levels of the cytokines IFN-γ, IL-2, IL-10, and IL-4 expressed by spleen mononuclear cells from mice immunized intranasally (i.n.) or subcutaneously (s.c.) with gp120 with or without mLT (LT(R192G)), and with or without an i.p. boost. See text of Section 7.2 for details.

5. DETAILED DESCRIPTION OF THE INVENTION

The present invention encompasses a composition and methods for its use to promote the production of mucosal and serum antibodies and cell mediated immune responses against

5 antigens that are administered simultaneously with a genetically modified bacterial toxin. The mode of administration may be, by way of example and not by way of limitation, by an intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, transdermal, epidural, pulmonary,

10 oral, nasal, gastric, intestinal, rectal, vaginal, or urethral route. Preferably, the route of administration is a mucosal route of administration, i.e., through a mucosal membrane or surface, such as an oral, nasal, gastric, intestinal, rectal, vaginal or urethral route. More preferably, the mucosal route

15 of administration is through an oral or nasal membrane. The modified toxin is a form of the heat-labile enterotoxin (LT) of E. coli which through genetic engineering has lost its trypsin sensitive site, rendering the molecule non-toxic at adjuvant effective doses, but yet, unexpectedly, retains its

20 ability to act as an immunological adjuvant. The mutant LT is herein termed "mLT". The invention is based on the discovery that mLT is as effective as LT as an immunological adjuvant, an unexpected and surprising result. mLT no longer has the enzymatic activity of ADP-ribosylation because the A subunit

25 can no longer be proteolytically processed. In contrast to published studies of Lycke and colleagues, which made it clear that alterations that affect the ADP-ribosylating activity of LT also prevent the molecule from functioning as an immunologic adjuvant [Lycke et al., 1992, Eur. J. Immunol.

30 22:2277-2281], the presently described mLT retains activity as an immunological adjuvant although, as demonstrated in the examples, it does not have in vitro ADP-ribosylating activity and has no enterotoxicity at an adjuvant effective dose.

The novel mutant form of the heat-labile enterotoxin

35 of E. coli, mLT, described herein, behaves as an adjuvant and elicits the production of both serum IgG and mucosal sIgA and cell mediated immune responses against antigens with which it

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is delivered. The utility of this surprising discovery is that
an adjuvant effective amount of mLT may be utilized in an
effective immunization program against a variety of pathogens
involving the administration of an effective amount of mLT
5 adjuvant in admixture with killed or attenuated pathogens or
relevant virulence determinants of specific pathogens with no
fear of the real or potential toxic side-effects associated
with oral administration of CT or LT.

        The present invention supersedes the prior art in that
10 the present invention may be used in a variety of
immunological applications in which CT, LT, or subunits of CT
or LT may have been used. With the use of mLT, however, there
are no real or potential side-effects, such as diarrhea,
associated with its use. In contrast to LT, which although not
15 toxic when first isolated from the bacterium, has the
potential to be fully toxic when exposed to proteases such as
those found in the mammalian intestine, mLT does not have the
potential to become toxic due to proteolytic activation.

        Another embodiment of the present invention is as a
20 component of a vaccine against enterotoxic organisms which
express cholera-like toxins. The present inventors have shown
that mLT is not subject to orally induced immune tolerance
when administered (see below), therefore mLT can function and
is highly desired as a component of vaccines directed against
25 enterotoxic organisms. Current technology provides for
vaccines against cholera-like toxin expressing organisms
containing killed whole cells and the B subunit of the toxin.
By replacing the B subunit with mLT in the vaccine, the
vaccine is improved in two different ways. First, mLT, which
30 has both the A and B subunits will now induce an immune
response not only to the B subunit but to the A subunit as
well. This provides for more epitopes for effective
neutralization. Second, the adjuvant activity inherent in mLT
will enhance the immune response against the killed whole cell
35 component of the vaccine.

        Further, other investigators [Häse et al., 1994,
Infect. Immun. 62:3051-3057] have shown that the A subunit,

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modified so that it is no longer toxic by altering the active
site of the ADP-ribosylating enzymatic activity, (as opposed
to the proteolytic site which is the subject of the current
invention) can induce an immune response against the wild type
5 A subunit. However, the A subunit so modified now lacks
immunologic adjuvant activity and is therefore less desirable
as a vaccine component than mLT.

Moreover, since antibodies against mLT cross-react
with LT and CT, mLT can be used in vaccines directed against
10 many types of enterotoxic bacterial organisms that express
cholera-like toxins, such as Escherichia spp. and Vibrio spp.

5.1. PRODUCTION OF mLT

The wild-type LT toxin is encoded on a naturally
15 occurring plasmid found in strains of enterotoxigenic E. coli
capable of producing this toxin. The present inventors had
previously cloned the LT gene from a human isolate of E. coli
designated H10407. This subclone consists of a 5.2 kb DNA
fragment from the enterotoxin plasmid of H10407 inserted into
20 the PstI site of plasmid pBR322 [Clements et al., 1983,
Infect. Immun. 40:653]. This recombinant plasmid, designated
pDF82, has been extensively characterized and expresses LT
under control of the native LT promoter. The next step in this
process was to place the LT gene under the control of a strong
25 promoter, in this case the lac promoter on plasmid pUC18. This
was accomplished by isolating the genes for LT-A and LT-B
separately and recombining them in a cassette in the vector
plasmid. This was an important step because it permitted
purification of reasonable quantities of LT and derived
30 mutants for subsequent analysis. This plasmid, designated
pBD94, is shown diagrammatically in Figure 1.

Both CT and LT are synthesized with a trypsin
sensitive peptide bond that joins the A_1 and A_2 pieces. This
peptide bond must be nicked for the molecule to be "toxic".
35 This is also true for diphtheria toxin, the prototypic A-B
toxin, and for a variety of other bacterial toxins. If the
A_1-A_2 bond of CT or LT is not removed, either by bacterial

proteases or intestinal proteases in the lumen of the bowel,
the A₁ piece cannot reach its target on the basolateral
surface of the intestinal epithelial cell. In contrast to CT,
LT is not fully biologically active when first isolated from
5 the cell. LT also requires proteolysis to be fully active and
the proteolytic activation does not occur inside of the
bacterium. Therefore, one means of altering the toxicity of
the molecule without affecting the NAD-binding site associated
with ADP-ribosylating enzymatic activity would be to remove by
10 genetic manipulation the trypsin sensitive amino acids that
join the A₁ and A₂ components of the A subunit. If the molecule
cannot be proteolytically cleaved, it will not be toxic. One
skilled in the art would predict that the molecule should,
however, retain its ADP-ribosylating enzymatic activity and
15 consequently, its adjuvant function.

      Figure 1 shows the sequence of the disulfide subtended
region that separates the A₁ and A₂ pieces. Within this region
is a single Arginine residue which is believed to be the site
of cleavage necessary to activate the toxic properties of the
20 molecule. This region was changed by site-directed mutagenesis
in such a way as to render the molecule insensitive to
proteolytic digestion and, consequently, nontoxic.

      Site-directed mutagenesis is accomplished by
hybridizing to single stranded DNA a synthetic oligonucleotide
25 which is complementary to the single stranded template except
for a region of mismatch near the center. It is this region
that contains the desired nucleotide change or changes.
Following hybridization with the single stranded target DNA,
the oligonucleotide is extended with DNA polymerase to create
30 a double stranded structure. The nick is then sealed with DNA
ligase and the duplex structure is transformed into an E. coli
host. The theoretical yield of mutants using this procedure is
50% due to the semi-conservative mode of DNA replication. In
practice, the yield is much lower. There are, however, a
35 number of methods available to improve yield and to select for
oligonucleotide directed mutants. The system employed utilized

a second mutagenic oligonucleotide to create altered
restriction sites in a double mutation strategy.

The next step was to substitute another amino acid for
Arg (i.e., GGA = Gly replaces AGA = Arg), thus preserving the
5 reading frame while eliminating the proteolytic site. mLT was
then purified by agarose affinity chromatography from one
mutant (pBD95) which had been confirmed by sequencing.
Alternate methods of purification will be apparent to those
skilled in the art. This mutant LT, designated LT($_{R192G}$) was
10 then examined by SDS-polyacrylamide gel electrophoresis for
modification of the trypsin sensitive bond. Samples were
examined with and without exposure to trypsin and compared
with native (unmodified) LT. mLT does not dissociate into A_1
and A_2 when incubated with trypsin, thereby indicating that
15 sensitivity to this protease this has been removed.

5.2.   MODE OF ADMINISTRATION OF mLT AND UNRELATED ANTIGENS
In accordance with the present invention, mLT can be
administered in conjunction with any biologically relevant
20 antigen and/or vaccine, such that an increased immune response
to said antigen and/or vaccine is achieved. In a preferred
embodiment, the mLT and antigen are administered
simultaneously in a pharmaceutical composition comprising an
effective amount of mLT and an effective amount of antigen.
25 The mode of administration may be, by way of example and not
by way of limitation, by an intradermal, intramuscular,
intraperitoneal, intravenous, subcutaneous, transdermal,
epidural, pulmonary, oral, nasal, gastric, intestinal, rectal,
vaginal, or urethral route. Preferably, the route of
30 administration is a mucosal route of administration, i.e.,
through a mucosal membrane or surface, such as an oral, nasal,
gastric, intestinal, rectal, vaginal or urethral route. More
preferably, the mucosal route of administration is through an
oral or nasal membrane.
35   The respective amounts of mLT and antigen will vary
depending upon the identity of the antigen employed and the
species of animal to be immunized. In one embodiment, the

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initial administration of mLT and antigen is followed by a
boost of the relevant antigen. In another embodiment no boost
is given. The timing of boosting may vary, depending on the
antigen and the species being treated. The modifications in
5 dosage range and timing of boosting for any given species and
antigen are readily determinable by routine experimentation.
The boost may be of antigen alone or in combination with mLT.
The mode of administration of the boost may either be oral,
nasal, or parenteral.

10   The methods and compositions of the present invention
are intended for use both in immature and mature vertebrates,
in particular birds, mammals, and humans. Useful antigens, as
examples and not by way of limitation, would include antigens
from pathogenic strains of bacteria (Streptococcus pyogenes,
15 Streptococcus pneumoniae, Neisseria gonorrhoea, Neisseria
meningitidis, Corynebacterium diphtheriae, Clostridium
botulinum, Clostridium perfringens, Clostridium tetani,
Haemophilus influenzae, Klebsiella pneumoniae, Klebsiella
ozaenae, Klebsiella rhinoscleromotis, Staphylococcus aureus,
20 Vibrio cholerae, Escherichia coli, Pseudomonas aeruginosa,
Campylobacter (Vibrio) fetus, Campylobacter jejuni, Aeromonas
hydrophila, Bacillus cereus, Edwardsiella tarda, Yersinia
enterocolitica, Yersinia pestis, Yersinia pseudotuberculosis,
Shigella dysenteriae, Shigella flexneri, Shigella sonnei,
25 Salmonella typhimurium, Treponema pallidum, Treponema
pertenue, Treponema carateneum, Borrelia vincentii, Borrelia
burgdorferi, Leptospira icterohemorrhagiae, Mycobacterium
tuberculosis, Toxoplasma gondii, Pneumocystis carinii,
Francisella tularensis, Brucella abortus, Brucella suis,
30 Brucella melitensis, Mycoplasma spp., Rickettsia prowazeki,
Rickettsia tsutsugumushi, Chlamydia spp., Helicobacter pylori;
pathogenic fungi (Coccidioides immitis, Aspergillus fumigatus,
Candida albicans, Blastomyces dermatitidis, Cryptococcus
neoformans, Histoplasma capsulatum); protozoa (Entomoeba
35 histolytica, Trichomonas tenas, Trichomonas hominis,
Trichomonas vaginalis, Trypanosoma gambiense, Trypanosoma
rhodesiense, Trypanosoma cruzi, Leishmania donovani,

Leishmania tropica, _Leishmania braziliensis_, _Pneumocystis pneumonia_, _Plasmodium vivax_, _Plasmodium falciparum_, _Plasmodium malaria_); or Helminths (_Enterobius vermicularis_, _Trichuris trichiura_, _Ascaris lumbricoides_, _Trichinella spiralis_,
5 _Strongyloides stercoralis_, _Schistosoma japonicum_, _Schistosoma mansoni_, _Schistosoma haematobium_, and hookworms) either presented to the immune system in whole cell form or in part isolated from media cultures designed to grow said organisms which are well know in the art or relevant antigens from said
10 organisms obtained by genetic engineering techniques or by chemical synthesis.

Other relevant antigens would be pathogenic viruses (as examples and not by limitation: Poxviridae, Herpesviridae, Herpes Simplex virus 1, Herpes Simplex virus 2, Adenoviridae,
15 Papovaviridae, Enteroviridae, Picornaviridae, Parvoviridae, Reoviridae, Retroviridae, influenza viruses, parainfluenza viruses, mumps, measles, respiratory syncytial virus, rubella, Arboviridae, Rhabdoviridae, Arenaviridae, Hepatitis A virus, Hepatitis B virus, Hepatitis C virus, Hepatitis E virus, Non-
20 A/Non-B Hepatitis virus, Rhinoviridae, Coronaviridae, Rotoviridae, and Human Immunodeficiency Virus) either presented to the immune system in whole or in part isolated from media cultures designed to grow such viruses which are well known in the art or relevant antigens therefrom obtained
25 by genetic engineering techniques or by chemical synthesis.

Further examples of relevant antigens include, but are not limited to, vaccines. Examples of such vaccines include, but are not limited to, influenza vaccine, pertussis vaccine, diphtheria and tetanus toxoid combined with pertussis vaccine,
30 hepatitis A vaccine, hepatitis B vaccine, hepatitis C vaccine, hepatitis E vaccine, Japanese encephalitis vaccine, herpes vaccine, measles vaccine, rubella vaccine, mumps vaccine, mixed vaccine of measles, mumps and rubella, papillomavirus vaccine, parvovirus vaccine, respiratory syncytial virus
35 vaccine, Lyme disease vaccine, polio vaccine, malaria vaccine, varicella vaccine, gonorrhea vaccine, HIV vaccine, schistosomiasis vaccine, rotavirus vaccine, mycoplasma

vaccine, pneumococcal vaccine, meningococcal vaccine and
others. These can be produced by known common processes. In
general, such vaccines comprise either the entire organism or
virus grown and isolated by techniques well known to the
5 skilled artisan or comprise relevant antigens of these
organisms or viruses which are produced by genetic engineering
techniques or chemical synthesis. Their production is
illustrated by, but not limited to, as follows:

Influenza vaccine: a vaccine comprising the whole or
10 part of hemagglutinin, neuraminidase, nucleoprotein and matrix
protein which are obtainable by purifying a virus, which is
grown in embryonated eggs, with ether and detergent, or by
genetic engineering techniques or chemical synthesis.

Pertussis vaccine: a vaccine comprising the whole or a
15 part of pertussis toxin, hemagglutinin and K-agglutinin which
are obtained from avirulent toxin (with formalin) which is
extracted by salting-out or ultracentrifugation from the
culture broth or bacterial cells of Bordetella pertussis, or
by genetic engineering techniques or chemical synthesis.

20   Diphtheria and tetanus toxoid combined with pertussis
vaccine: a vaccine mixed with pertussis vaccine, diphtheria
and tetanus toxoid.

Japanese encephalitis vaccine: a vaccine comprising
the whole or part of an antigenic protein which is obtained by
25 culturing a virus intracerebrally in mice and purifying the
virus particles by centrifugation or ethyl alcohol and
inactivating the same, or by genetic engineering techniques or
chemical synthesis.

Hepatitis B vaccine: a vaccine comprising the whole or
30 part of an antigen protein which is obtained by isolating and
purifying the HBs antigen by salting-out or
ultracentrifugation, obtained from hepatitis carrying blood,
or by genetic engineering techniques or by chemical synthesis.

Measles vaccine: a vaccine comprising the whole or
35 part of a virus grown in a cultured chick embryo cells or
embryonated egg, or a protective antigen obtained by genetic
engineering or chemical synthesis.

Rubella vaccine: a vaccine comprising the whole or part of a virus grown in cultured chick embryo cells or embryonated egg, or a protective antigen obtained by genetic engineering techniques or chemical synthesis.

5   Mumps vaccine: a vaccine comprising the whole or part of a virus grown in cultured rabbit cells or embryonated egg, or a protective antigen obtained by genetic engineering techniques or chemical synthesis.

Mixed vaccine of measles, rubella and mumps: a vaccine 10 produced by mixing measles, rubella and mumps vaccines.

Rota vaccine: a vaccine comprising the whole or part of a virus grown in cultured MA 104 cells or isolated from the patient's feces, or a protective antigen obtained by genetic engineering techniques or chemical synthesis.

15   Mycoplasma vaccine: a vaccine comprising the whole or part of mycoplasma cells grown in a liquid culture medium for mycoplasma or a protective antigen obtained by genetic engineering techniques or chemical synthesis.

Those conditions for which effective prevention may be 20 achieved by the present method will be obvious to the skilled artisan.

The vaccine preparation compositions of the present invention can be prepared by mixing the above illustrated antigens and/or vaccines with mLT at a desired ratio. The 25 preparation should be conducted strictly aseptically, and each component should also be aseptic. Pyrogens or allergens should naturally be removed as completely as possible. The antigen preparation of the present invention can be used by preparing the antigen *per se* and the mLT separately.

30   Further, the present invention encompasses a kit comprising an effective amount of antigen and an adjuvant effective amount of mLT. In use, the components of the kit can either first be mixed together and then administered orally or the components can be administered orally separately 35 within a short time of each other.

The vaccine preparation compositions of the present invention can be combined with either a liquid or solid

pharmaceutical carrier, and the compositions can be in the form of tablets, capsules, powders, granules, suspensions or solutions. The compositions can also contain suitable preservatives, coloring and flavoring agents, or agents that 5 produce slow release such as encapsulating agents such as liposomes, microparticles, microcapsules, etc.   Potential carriers that can be used in the preparation of the pharmaceutical compositions of this invention include, but are not limited to, gelatin, gelatin capsules, sugars, cellulose 10 derivations such as sodium carboxymethyl cellulose, talc, magnesium stearate, vegetable oil such as peanut oil, etc., glycerin, glycerol, sorbitol, agar, water, saline, buffered saline, sterile isotonic aqueous buffer, and combinations thereof.   Carriers may also serve as a binder to facilitate 15 tabletting of the compositions for convenient oral administration.   The carrier is preferably sterile.   The formulation should suit the mode of administration.

The composition, if desired, can also contain minor amounts of wetting or emulsifying agents, or pH buffering 20 agents.   The composition can be a liquid solution, suspension, emulsion, tablet, pill, capsule, sustained release formulation, or powder.   Oral formulation can include standard carriers such as pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, 25 magnesium carbonate, etc.

The mode of administration of the vaccine preparation compositions of the invention include, but are not limited to an intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, transdermal, epidural, pulmonary, oral, nasal, 30 gastric, intestinal, rectal, vaginal, or urethral route. Preferably, the route of administration is a mucosal route of administration, i.e., through a mucosal membrane or surface, such as an oral, nasal, gastric, intestinal, rectal, vaginal or urethral route.   More preferably, the mucosal route of 35 administration is through an oral or nasal membrane.

The compounds may be administered in any convenient manner, for example by infusion or bolus injection, by

absorption through epithelial or mucocutaneous linings (e.g., oral mucosa, rectal and intestinal mucosa, etc.) and may be administered together with other biologically active agents. Administration can be systemic or local. Pulmonary

5 administration can also be employed, e.g., by use of an inhaler or nebulizer, and formulation with an aerosolizing agent.

In a specific embodiment, it may be desirable to administer the pharmaceutical compositions of the invention

10 locally to the area in need of treatment; this may be achieved, by way of example and not by way of limitation, by topical application, by injection, by means of a catheter, by means of a suppository, or by means of an implant, the implant being of a porous, non-porous, or gelatinous material,

15 including membranes, such as sialastic membranes, or fibers.

In another embodiment, the pharmaceutical compositions of the invention can be delivered in a vesicle, in particular a liposome [see Langer, 1990, Science 249:1527-1533; Treat et al., 1989, in *Liposomes in the Therapy of Infectious Disease*

20 and Cancer, Lopez-Berestein and Fidler (eds.), Liss, New York, pp. 353-365; Lopez-Berestein, *ibid.*, pp. 317-327; see generally *ibid.*].

In yet another embodiment, the pharmaceutical compositions of the invention can be delivered in a controlled

25 release system. In one embodiment, a pump may be used [see Langer, *supra*; Sefton, 1987, CRC Crit. Ref. Biomed. Eng. 14: 201; Buchwald et al., 1980, Surgery 88: 507; Saudek et al., 1989, N. Engl. J. Med. 321: 574]. In another embodiment, polymeric materials can be used [see 1974, *Medical*

30 *Applications of Controlled Release*, Langer and Wise (eds.), CRC Pres., Boca Raton, Florida; 1984, *Controlled Drug Bioavailability, Drug Product Design and Performance*, Smolen and Ball (eds.), Wiley, New York; Ranger and Peppas, 1983, J. Macromol. Sci. Rev. Macromol. Chem. 23:61; see also Levy et

35 al., 1985, Science 228: 190; During et al., 1989, *Ann. Neurol.* 25: 351; Howard et al., 1989, J. Neurosurg. 71: 105].

In yet another embodiment, a controlled release system
can be placed in proximity of a mucosal target, thus requiring
only a fraction of the systemic dose [see, e.g., Goodson,
1984, in Medical Applications of Controlled Release, supra,
5 vol. 2, pp. 115-138].

Other controlled release systems are discussed in the
review by Langer [1990, Science 249: 1527-1533].

The vaccine preparation compositions of this invention
may be maintained in a stable storage form for ready use by
10 lyophilization or by other means well known to those skilled
in the art.

In one embodiment of the invention, a vaccine
preparation composition of the invention is administered
orally. For oral administration, the vaccine preparation may
15 be reconstituted as a suspension in buffered saline, milk, or
any other physiologically compatible liquid medium. The
medium may be made more palatable by the addition of suitable
coloring and flavoring agents as desired.

Oral administration of the vaccine preparation
20 compositions may be preceded by an oral dosage of an effective
amount of a gastric acid neutralizing agent. While many
compounds could be used for this purpose, sodium bicarbonate
is preferred. Alternatively, the vaccine compositions may be
delivered in enteric coated capsules (i.e., capsules that
25 dissolve only after passing through the stomach).

The ingredients can be supplied either separately or
mixed together in unit dosage form, for example, as a dry
lyophilized powder or water free concentrate in a
hermetically sealed container such as an ampoule or sachette
30 indicating the quantity of active agent. Where the
composition is administered by injection, an ampoule of
sterile diluent can be provided so that the ingredients may
be mixed prior to administration.

In a specific embodiment, a lyophilized mixture of an
35 effective amount of antigen and an adjuvant effective amount
of mLT of the invention is provided in a first container; a
second container comprises diluent consisting of an aqueous

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solution of 50% glycerin, 0.25% phenol, and an antiseptic
(e.g., 0.005% brilliant green).

       The precise dose of vaccine preparation to be employed
in the formulation will also depend on the route of
5 administration, and the nature of the patient, and should be
decided according to the judgment of the practitioner and
each patient's circumstances according to standard clinical
techniques. An effective immunizing amount is that amount
sufficient to produce an immune response to the antigen in
10 the host to which the vaccine preparation is administered.

       Use of purified antigens as vaccine preparations can
be carried out by standard methods. For example, the
purified antigens should be adjusted to an appropriate
concentration, formulated with an adjuvant-effective amount
15 of mLT and packaged for use. The immunogen may also be
incorporated into liposomes, or conjugated to polysaccharides
and/or other polymers for use in a vaccine formulation.

       The following examples are presented for purposes of
20 illustration only and are not intended to limit the scope of
the invention in any way.

       6.   EXAMPLE 1: CHARACTERIZATION OF mLT ACTIVITIES
              6.1.   CONSTRUCTION OF mLT
25   The wild-type LT toxin is encoded on a naturally
occurring plasmid found in strains of enterotoxigenic E. coli
capable of producing this toxin. The present inventors had
previously cloned the LT gene from a human isolate of E. coli
designated H10407. This subclone consists of a 5.2 kb DNA
30 fragment from the enterotoxin plasmid of H10407 inserted into
the PstI site of plasmid pBR322 [Clements et al., 1983,
Infect. Immun. 40:653]. This recombinant plasmid, designated
pDF82, has been extensively characterized and expresses LT
under control of the native LT promoter. The next step in
35 this process was to place the LT gene under the control of a
strong promoter, in this case the lac promoter on plasmid
pUC18. This was accomplished by isolating the genes for LT-A

and LT-B separately and recombining them in a cassette in the vector plasmid. This was an important step because it permitted purification of reasonable quantities of LT and derived mutants for subsequent analysis. This plasmid,
5 designated pBD94, is shown diagrammatically in Figure 1.

Both CT and LT are synthesized with a trypsin sensitive peptide bond that joins the A_1 and A_2 pieces. This peptide bond must be nicked for the molecule to be "toxic". This is also true for diphtheria toxin, the prototypic A-B
10 toxin, and for a variety of other bacterial toxins. If the A_1-A_2 bond is not removed, either by bacterial proteases or intestinal proteases in the lumen of the bowel, i.e., proteolytic processing or activation, the A_1 piece cannot reach its target on the basolateral surface of the intestinal
15 epithelial cell. In contrast to CT, LT is not fully biologically active when first isolated from the cell. LT also requires proteolysis to be fully active and the proteolytic activation does not occur inside of the bacterium. Therefore, one means of altering the toxicity of
20 the molecule without affecting the ADP-ribosylating enzymatic activity would be to remove by genetic manipulation the trypsin sensitive amino acids that join the A_1 and A_2 components of the A subunit. If the molecule cannot be proteolytically cleaved, it will not be toxic. One skilled in
25 the art would predict that the molecule should, however, retain its ADP-ribosylating enzymatic activity and consequently, its adjuvant function.

Figure 1 shows the sequence of the disulfide subtended region that separates the A_1 and A_2 pieces. Within this region
30 is a single Arginine residue which is believed to be the site of cleavage necessary to activate the toxic properties of the molecule. This region was changed by site-directed mutagenesis in such a way as to render the molecule insensitive to proteolytic digestion and, consequently,
35 nontoxic.

Site-directed mutagenesis is accomplished by hybridizing to single stranded DNA a synthetic

oligonucleotide which is complementary to the single stranded template except for a region of mismatch near the center. It is this region that contains the desired nucleotide change or changes. Following hybridization with the single stranded

5 target DNA, the oligonucleotide is extended with DNA polymerase to create a double stranded structure. The nick is then sealed with DNA ligase and the duplex structure is   ⌐ transformed into an E. coli host. The theoretical yield of mutants using this procedure is 50% due to the

10 semi-conservative mode of DNA replication. In practice, the yield is much lower. There are, however, a number of methods available to improve yield and to select for oligonucleotide directed mutants. The system employed utilized a second mutagenic oligonucleotide to create altered restriction sites

15 in a double mutation strategy.

The next step was to substitute another amino acid for Arg (i.e., GGA = Gly replaces AGA = Arg), thus preserving the reading frame while eliminating the proteolytic site. mLT was then purified by agarose affinity chromatography from one

20 mutant (pBD95) which had been confirmed by sequencing. Alternate methods of purification will be apparent to those skilled in the art. This mutant LT, designated LT($R192G$) was then examined by SDS-polyacrylamide gel electrophoresis for modification of the trypsin sensitive bond. Samples were

25 examined with and without exposure to trypsin and compared with native (unmodified) LT. mLT does not dissociate into A_1 and A_2 when incubated with trypsin, thereby indicating that sensitivity to protease has been removed.

30   6.2.   EFFECT OF mLT ON Y-1 ADRENAL CELLS

It would be predicted by one skilled in the art that mLT would not be active in the Y-1 adrenal cell assay. This prediction would be based upon previous findings [Clements and Finkelstein, 1979, Infect. Immun. 24:760-769] that

35 un-nicked LT was more than 1,000 fold less active in this assay system than was CT and that trypsin treatment activated LT to the same level of biological activity as CT in this

assay. It was presumed that the residual activity of LT
observed in this assay in the absence of trypsin activation
was a function of some residual protease activity which could
not be accounted for. For instance, trypsin is used in the
5 process of subculturing Y-1 adrenal cells. It was therefore
assumed that LT that could not be nicked would be completely
inactive in the Y-1 adrenal cell assay. Results are shown in
Table I.

10

15

20

25

30

35

TABLE I

Toxin	Trypsin Activated	Specific Activity [a]
Cholera Toxin	−	15
LT	−	60
LT	+	15
LT$_{(R192G)}$	−	48,800
LT$_{(R192G)}$	+	48,800

[a]Minimum dose (picograms per well) required to produce (>50%) cell rounding.

Table I demonstrates the <u>unexpected finding</u> that mLT retained a basal level of activity in the Y-1 adrenal cell assay even though it could not be proteolytically processed. As shown in Table I, CT and native LT treated with trypsin have the same level of activity (15 pg) on Y-1 adrenal cells. By contrast, mLT (48,000 pg) was >1,000-fold less active than CT or native LT and could not be activated by trypsin. The residual basal activity undoubtedly reflects a different and heretofore unknown pathway of adrenal cell activation than that requiring separation of the A_1 - A_2 linkage.

6.3.   ADP-RIBOSYLATING ENZYMATIC ACTIVITY OF mLT

Because the mutation replacing Arg_{192} with Gly_{192} does not alter the enzymatic site of the A_1 moiety, one skilled in the art would predict that mLT would retain its ADP-ribosylating enzymatic activity. To examine this property, the NAD-Agmatine ADP-ribosyltransferase Assay was employed [Moss et al., 1993, J. Biol. Chem. 268:6383-6387]. As shown in Figure 2, CT produces a dose-dependent increase in the levels of ADP-ribosylagmatine, a function of the ADP-ribosyltransferase activity of this molecule.

TABLE II

ADP-Ribosyltransferase Activity of CT, native LT, and LT$_{(R192G)}$					
Experiment	1	2	3	4	Mean±SEM
No Toxin	ND	9.12	5.63	14.17	9.64±2.48
1μgCT	ND	17.81	17.60	25.75	20.39±2.68
10μgCT	ND	107.32	111.28	104.04	107.55±2.09
100μgCT	351.55	361.73	308.09	ND	340.46±16.45
100μgLT	17.32	14.48	13.86	ND	15.22±1.07
100μgLT +Trypsin	164.10	189.89	152.96	ND	168.98±10.94
100μg LT$_{(R192G)}$	14.58	12.34	9.30	ND	12.07±1.53
100μg LT$_{(R192G)}$ +Trypsin	14.73	8.90	10.47	ND	11.37±1.74
ND= Not Done data expressed in fMoles min^{-1}					

Table II demonstrates in tabular form the <u>unexpected</u>
<u>finding</u> that mLT lacked any detectable ADP-ribosylating
enzymatic activity, with or without trypsin activation, even
though the enzymatic site had not been altered and there was
a demonstratable basal level of activity in the Y-1 adrenal
cell assay.

6.4. ENTEROTOXIC ACTIVITY OF mLT

Because of the unexpected finding that mLT lacks any
detectable ADP-ribosylating enzymatic activity, with or
without trypsin activation, even though the enzymatic site
has not been altered and the additional finding that there is
a basal level of activity in the Y-1 adrenal cell assay, it
was unclear whether mLT would retain any of its enterotoxic
properties. An ideal adjuvant formulation of mLT would
retain its ability to act as an immunological adjuvant but

would lack the real or potential side-effects, such as
diarrhea, associated with the use of LT or CT. Figure 3
demonstrates that mLT does not induce net fluid secretion in
the patent mouse model, even at a dose of 125 μg. This dose
5 is more than five times the adjuvant effective dose for LT in
this model. Importantly, the potential toxicity of native LT
can be seen at this level.

6.5. ADJUVANT ACTIVITY OF mLT

10   One skilled in the art would predict that since mLT
possessed no demonstrable ADP-ribosyltransferase activity and
is not enterotoxic, it would lack adjuvant activity. This
prediction would be based upon the report by Lycke et al.
[Lycke et al., 1992, Eur. J. Immunol. 22:2277-2281] where it
15 is made clear that alterations that affect the
ADP-ribosylating enzymatic activity of the toxin and alter
the ability to increase intracellular levels of cAMP also
prevent the molecule from functioning as an adjuvant. As
demonstrated above, mLT has no ADP-ribosylating enzymatic
20 activity and only some undefined basal activity in Y-1
adrenal cells, and induces no net fluid secretion in the
patent mouse model.
          In order to examine the adjuvant activity of mLT the
following experiment was performed. Three groups of BALB/c
25 mice were immunized. Animals were inoculated intragastrically
with a blunt tipped feeding needle (Popper & Sons, Inc., New
Hyde Park, New York). On day 0, each group was immunized
orally as follows: Group A received 0.5 ml of PBS containing
5 mg of OVA, Group B received 0.5 ml of PBS containing 5 mg
30 of OVA and 25 μg of native LT, and Group C received 0.5 ml of
PBS containing 5 mg of OVA and 25 μg of mLT. Each regimen was
administered again on days 7 and 14. On day 21, all animals
were boosted i.p. with 1 μg of OVA in 20% Maalox. One week
after the i.p. inoculation animals were sacrificed and
35 assayed for serum IgG and mucosal IgA antibodies directed
against OVA and LT by ELISA.

Reagents and antisera for the ELISA were obtained from
Sigma Chemical Co. Samples for ELISA were serially diluted in
phosphate buffered saline (pH 7.2)-0.05% Tween 20
(PBS-TWEEN). For anti-LT determinations, microtiter plates

5 were precoated with 1.5 μg per well of mixed gangliosides
(Type III), then with 1 μg per well of purified LT. Anti-OVA
responses were determined on microtiter plates precoated with
10 μg per well of OVA. Serum anti-LT and anti-OVA were
determined with rabbit antiserum against mouse IgG conjugated

10 to alkaline phosphatase. Mucosal anti-LT and anti-OVA IgA
were assayed with goat antiserum against mouse IgA
[alpha-chain specific] followed by rabbit antiserum against
goat IgG conjugated to alkaline phosphatase. Reactions were
stopped with 3N NaOH. Values for IgG and IgA were determined

15 from a standard curve with purified mouse myeloma proteins
(MOPC 315, γA(IgAλ2); MOPC 21, γG1: Litton Bionetics, Inc.,
Charleston, SC).

6.5.1.   SERUM IgG ANTI-OVA

20   As shown in the Figure 4A, animals immunized orally
with OVA and LT developed a significantly higher serum IgG
anti-OVA response following subsequent parenteral
immunization with OVA (4,058 μg/ml) than those immunized
orally with OVA alone and subsequently immunized parenterally

25 with OVA (No detectable anti-OVA response) (Student t-test p=
.031). Significantly, animals immunized orally with OVA and
mLT also developed a significantly higher serum IgG anti-OVA
response following subsequent parenteral immunization with
OVA (1,338 μg/ml) than those immunized orally with OVA alone

30 and subsequently immunized parenterally with OVA (No
detectable anti-OVA response) (Student t-test p= .0007).

6.5.2.   MUCOSAL sIgA ANTI-OVA
As shown in the Figure 4B, similar results were
35 obtained when anti-OVA IgA responses were compared within
these same groups of animals. Animals immunized orally with
OVA and LT developed a significantly higher mucosal IgA

anti-OVA response following subsequent parenteral
immunization with OVA (869 ng/ml) than those immunized orally
with OVA alone and subsequently immunized parenterally with
OVA (No detectable anti-OVA response) (Student t-test p=
5 .0131). As above, animals immunized orally with OVA _and_ mLT
also developed a significantly higher mucosal IgA anti-OVA
response following subsequent parenteral immunization with
OVA (230 ng/ml) than those immunized orally with OVA alone
and subsequently immunized parenterally with OVA (No
10 detectable anti-OVA response) (Student t-test p= .0189).

6.5.3.   SERUM IgG ANTI-LT

The ability of LT and mLT to elicit an anti-LT
antibody response in these same animals was also examined.
15 This was important in that it would provide an indication of
whether the mutant LT was able to prevent induction of
tolerance to itself in addition to functioning as an adjuvant
for other proteins. As shown in Figure 5A, animals immunized
orally with OVA _and_ LT developed a significantly higher serum
20 IgG anti-LT response following subsequent parenteral
immunization with OVA (342 µg/ml) than those immunized orally
with OVA alone and subsequently immunized parenterally with
OVA (No detectable anti-LT response) (Student t-test p=
.0005). Animals immunized orally with OVA _and_ mLT also
25 developed a significantly higher serum IgG anti-LT response
following subsequent parenteral immunization with OVA (552
µg/ml) than those immunized orally with OVA alone and
subsequently immunized parenterally with OVA (No detectable
anti-LT response) (Student t-test p= .0026).
30

6.5.4.   MUCOSAL sIgA ANTI-LT

As shown in the Figure 5B, similar results were
obtained when anti-LT IgA responses were compared within
these same groups of animals. Animals immunized orally with
35 OVA _and_ LT developed a significantly higher mucosal IgA
anti-LT response following subsequent parenteral immunization
with OVA (4,328 ng/ml) than those immunized orally with OVA

alone and subsequently immunized parenterally with OVA (No
detectable anti-LT response) (Student t-test p= .0047). As
above, animals immunized orally with OVA and mLT also
developed a significantly higher mucosal IgA anti-LT response
5 following subsequent parenteral immunization with OVA (1,463
ng/ml) than those immunized orally with OVA alone and
subsequently immunized parenterally with OVA (No detectable
anti-LT response) (Student t-test p= .0323).

10   7.   EXAMPLE 2: IMMUNIZATION USING HIV-1 COMPONENTS
            AS IMMUNOGENS AND mLT AS ADJUVANT

      7.1. IMMUNIZATION WITH HIV-1 VIRAL LYSATE
            A commercially available HIV-1 viral lysate
preparation was used an as immunogen in this study (Advanced
15 Biotechnologies, Inc., Columbus MD). The HIV-1 viral lysate
preparation was derived from tissue culture grown, sucrose
density purified HIV-1 IIIB inactivated with Triton X-100 and
heat. Analysis of this material indicated the presence of
the following structural proteins when reacted with HIV-1
20 positive human sera: p17, p24, p31, gp41, p51, p55, p66,
gp120 and gp160.
            Two groups of BALB/c mice were immunized i.n. with 10
μg HIV-1 viral lysate with or without mLT (LT(R192G)).
Animals were immunized four times without a parenteral boost
25 and humoral anti-HIV responses were analyzed. Individual
serum, nasal and vaginal samples were examined by ELISA for
antibodies directed against the viral lysate. Serum samples
were diluted 1:8 and vaginal and nasal washes were diluted
1:2 for ELISA analysis. The procedure used to collect
30 vaginal secretions results in the dilution of vaginal
secretions by a factor of 10 [Staats et al., 1996, J.
Immunol. 157:462-472]. Thus, antibody titers in undiluted
vaginal secretions are approximately 10-fold higher than
shown. Results are illustrated in Figure 6. Values reported
35 are the mean OD 405 nm for each group of animals.
            The results presented in Figure 6 show that animals
immunized i.n. with the HIV-1 viral lysate in conjunction

with mLT (LT(R192G)) had significantly higher anti-HIV serum
IgG, nasal and vaginal IgA antibody titers than did animals
immunized i.n. with HIV-1 viral lysate alone. These findings
are clearly important because they demonstrate the utility of
5 mLT as an adjuvant with HIV for development of both serum and
mucosal antibody responses.

7.2. IMMUNIZATION WITH HIV-1 gp120

The HIV-1 envelope glycoprotein gp120 has been the
10 subject of numerous vaccine studies in non-human primates and
humans. Immunization with gp120, either in combination with
various adjuvants or as expressed by live attenuated viral or
bacterial vectors, induces anti-retroviral CD4 T-cell
proliferative responses, anti-retroviral CD8 cytotoxic
15 lymphocytes (CTLs), and antibodies capable of neutralizing
laboratory adapted retrovirus strains [Haynes, 1996, Lancet
348:933-937]. However, Sullivan et al. [1995, J. Virol.
69:4413-4422] have recently shown that anti-recombinant gp120
antibodies raised in animals or in human volunteers
20 neutralize HIV grown in laboratory adapted T-cell lines but
not in primary isolates of the virus grown in peripheral
blood mononuclear cells (PBMCs). These findings, coupled
with the observation of Kahn et al. [J. Infect. Dis.
171:1343-1347] demonstrating that parenteral immunization
25 with gp120 did not protect against HIV-1 infection require
that other immunization strategies be explored.

For these studies, groups of BALB/C mice were
immunized with a commercially available HIV-1 recombinant
gp120 preparation (Advanced Biotechnologies, Inc., Columbus
30 MD). Mice were immunized i.n. or subcutaneously (s.c.) with
1 μg HIV-1 gp120 with or without mLT (LT(R192G)). Animals
were immunized i.n. or s.c. three times with or without an
i.p. boost and their humoral and cellular anti-gp120
responses analyzed.

35   Pooled serum and vaginal samples were examined by
ELISA for antibodies directed against gp120. Serum samples
were diluted 1:8 and vaginal washes diluted 1:2 for ELISA

analysis.   Values reported are OD 405 nm multiplied by the
appropriate dilution factor.   The procedure used to collect
vaginal secretions results in the dilution of vaginal
secretions by a factor of 10 [Staats et al., 1996, J.

5 Immunol. 157:462-472]. Thus, antibody titers in undiluted
vaginal secretions are approximately 10-fold higher than
shown.   Results are presented in Figures 7 and 8.
Mononuclear cells from the spleens of immunized animals were
analyzed for cytokine production in antigen restimulation

10 assays.   Results are presented in Figure 9.

Figure 7 shows that animals immunized i.n. or s.c.
with gp120 in conjunction with mLT (LT(R192G)) had
significantly higher anti-gp120 serum IgG antibody titers
than did animals immunized i.n. with gp120 alone.

15 Figure 8 shows that the highest vaginal anti-gp120 IgA
responses were observed in animals immunized i.n. with gp120
in conjunction with mLT and boosted i.p. with gp120. These
results show clearly that the magnitude and distribution of
the anti-gp120 antibody response can be influenced by the

20 route of immunization, use of mLT as an adjuvant, and
parenteral boosting following mucosal priming.

Figure 9 shows that there was a limited cytokine
response by spleen mononuclear cells from animals immunized
i.n. or s.c. with gp120 alone. By contrast, mononuclear

25 cells from animals immunized i.n. or s.c. with gp120 in
conjunction with mLT had significantly increased production
of antigen-specific IFN-γ, IL-2, and IL-10.

These findings are significant because they
demonstrate that both humoral and cellular anti-gp120

30 responses are elicited by immunization with gp120 in
conjunction with mLT, which can greatly facilitate the
induction of both T_H1 and T_H2 antigen-specific cytokine
responses. Moreover, the qualitative and quantitative
aspects of both the humoral and cellular response are

35 determined by the use of mLT as an adjuvant. The route of
immunization may also be important. Parenteral boosting
following mucosal priming may be advantageous.

- 40 -

8. DEPOSIT OF MICROORGANISMS

The following plasmid was deposited with the American
Type Culture Collection (ATCC), Rockville, MD on August 18,
1994, and has been assigned the indicated accession number:

5   Plasmid   Accession Number
        pBD95 in E. coli LTR192G   ATCC 69683

The invention described and claimed herein is not to
be limited in scope by the specific embodiments herein
10 disclosed since these embodiments are intended as
illustration of several aspects of the invention. Any
equivalent embodiments are intended to be within the scope of
this invention. Indeed, various modifications of the
invention in addition to those shown and described herein
15 will become apparent to those skilled in the art from the
foregoing description. Such modifications are also intended
to fall within the scope of the appended claims.

It is also to be understood that all base pair and
amino acid residue numbers and sizes given for nucleotides
20 and peptides are approximate and are used for purposes of
description.

A number of references are cited herein, the entire
disclosures of which are incorporated herein, in their
entirety, by reference.

25

30

35

SEQUENCE LISTING

(1) GENERAL INFORMATION:

   (i) APPLICANT: THE ADMINISTRATORS OF THE TULANE EDUCATIONAL FUND

   (ii) TITLE OF INVENTION: MUTANT ENTEROTOXIN EFFECTIVE AS A NON-TOXIC ADJUVANT FOR HIV

   (iii) NUMBER OF SEQUENCES: 5

   (iv) CORRESPONDENCE ADDRESS:
      (A) ADDRESSEE: PENNIE & EDMONDS LLP
      (B) STREET: 1155 Avenue of the Americas
      (C) CITY: New York
      (D) STATE: New York
      (E) COUNTRY: U.S.A.
      (F) ZIP: 10036

   (v) COMPUTER READABLE FORM:
      (A) MEDIUM TYPE: Floppy disk
      (B) COMPUTER: IBM PC compatible
      (C) OPERATING SYSTEM: PC-DOS/MS-DOS
      (D) SOFTWARE: PatentIn Release #1.0, Version #1.25

   (vi) CURRENT APPLICATION DATA:
      (A) APPLICATION NUMBER: PCT/US98/01546
      (B) FILING DATE: 26-JAN-1998
      (C) CLASSIFICATION:

   (viii) ATTORNEY/AGENT INFORMATION:
      (A) NAME: Misrock, S. Leslie
      (B) REGISTRATION NUMBER: 18,872
      (C) REFERENCE/DOCKET NUMBER: 5113-050-228

   (ix) TELECOMMUNICATION INFORMATION:
      (A) TELEPHONE: (212) 790-9090
      (B) TELEFAX: (212) 869-8864/9741
      (C) TELEX: 66141 PENNIE

   (2) INFORMATION FOR SEQ ID NO:1:

   (i) SEQUENCE CHARACTERISTICS:
      (A) LENGTH: 45 base pairs
      (B) TYPE: nucleic acid
      (C) STRANDEDNESS: single
      (D) TOPOLOGY: linear

   (ii) MOLECULE TYPE: DNA

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GGTTGTGGAG ATTCATCAAG AACAATTACA GGTGATACTT GTAAT   45

   (2) INFORMATION FOR SEQ ID NO:2:

   (i) SEQUENCE CHARACTERISTICS:
      (A) LENGTH: 15 amino acids
      (B) TYPE: amino acid
      (C) STRANDEDNESS:
      (D) TOPOLOGY: unknown

   (ii) MOLECULE TYPE: peptide

-42-

SUBSTITUTE SHEET (rule 26)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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Gly Cys Gly Asp Ser Ser Arg Thr Ile Thr Gly Asp Thr Cys Asn
1               5               10              15
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        (2) INFORMATION FOR SEQ ID NO:3:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 18 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

TCATCAGGAA CAATTACA   18

        (2) INFORMATION FOR SEQ ID NO:4:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 45 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GGTTGTGGAG ATTCATCAGG AACAATTACA GGTGATACTT GTAAT   45

        (2) INFORMATION FOR SEQ ID NO:5:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 15 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS:
        (D) TOPOLOGY: unknown

    (ii) MOLECULE TYPE: peptide

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

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Gly Cys Gly Asp Ser Ser Gly Thr Ile Thr Gly Asp Thr Cys Asn
1               5               10 -            15
```

SUBSTITUTE SHEET (rule 26)

MICROORGANISMS

Optional Sheet in connection with the microorganism referred to on page _41_, lines _1-25_ of the description *

A. IDENTIFICATION OF DEPOSIT *

Further deposits are identified on an additional sheet

Name of depositary institution *

American Type Culture Collection

Address of depositary institution (including postal code and country) *

12301 Parklawn Drive
Rockville, MD 20852
US

Date of deposit * _August 18, 1994_ Accession Number * _69683_

B. ADDITIONAL INDICATIONS * (leave blank if not applicable). This information is continued on a separate attached sheet

C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE * (if the indications are not all designated States)

D. SEPARATE FURNISHING OF INDICATIONS * (leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later * (Specify the general nature of the indications e.g., "Accession Number of Deposit")

E. ☒ This sheet was received with the International application when filed (to be checked by the receiving Office)

PAUL F. URRUTIA
(Authorized Officer)

☐ The date of receipt (from the applicant) by the International Bureau *

was _____
                                   (Authorized Officer)

Form PCT/RO/134 (January 1981)

CLAIMS:

1. A method of creating or sustaining a protective or adaptive immune response to an HIV virus in a host comprising
5 mucosally administering an admixture of an effective amount of an HIV antigen and an adjuvant effective amount of a mutant *E. coli* heat-labile enterotoxin holotoxin, in which arginine at amino acid position 192 is replaced with glycine, which holotoxin is substantially less toxic than native *E.*
10 *coli* heat-labile enterotoxin holotoxin as measured in the Y-1 adrenal cell assay and which has immunologic adjuvant activity but lacks ADP-ribosylating enzymatic activity as measured by the NAD-Agmatine ADP-ribosyltransferase Assay in an acceptable pharmaceutical carrier.
15

2. The method of claim 1 wherein an antibody response is produced.

3. The method of claim 2 wherein a serum response is
20 produced.

4. The method of claim 2 wherein a mucosal response is produced.

25   5. The method of claim 1 wherein a cell mediated response is produced.

6. The method of claim 1 further comprising administering a subsequent boost of the HIV antigen alone or
30 in combination with the adjuvant.

7. The method of claim 1 wherein the admixture is delivered in a single dose.

35   8. The method of claim 1 wherein the admixture is administered by a route of administration selected from the group consisting of intradermal, intramuscular,

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intraperitoneal, intravenous, subcutaneous, transdermal, epidural, pulmonary, oral, nasal, gastric, intestinal, rectal, vaginal, or urethral routes.

5   9.   The method of claim 1 wherein the HIV antigen is an HIV lysate.

   10. The method of claim 1 wherein the HIV antigen is a gp120 glycoprotein.

10

        11. A method of inducing a protective immune response to an HIV virus in a host comprising administering an effective amount of an HIV antigen in conjunction with an adjuvant effective amount of a mutant *E. coli* heat-labile
15 enterotoxin holotoxin, in which arginine at amino acid position 192 is replaced with glycine, which holotoxin is substantially less toxic than native *E. coli* heat-labile enterotoxin holotoxin as measured in the Y-1 adrenal cell assay and which has immunologic adjuvant activity but lacks
20 ADP-ribosylating enzymatic activity as measured by the NAD-Agmatine ADP-ribosyltransferase Assay.

        12. The method of claim 9 in which the HIV antigen and the holotoxin are administered simultaneously.

25

        13. The method of claim 9 in which the HIV antigen and the holotoxin are administered separately within a short time of each other.

30   14. The method of claim 9 wherein the HIV antigen is administered by a route of administration selected from the group consisting of intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, transdermal, epidural, pulmonary, oral, nasal, gastric, intestinal,
35 rectal, vaginal, or urethral routes.

15. The method of claim 9 wherein the holotoxin is administered by a route of administration selected from the group consisting of intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, transdermal,
5 epidural, pulmonary, oral, nasal, gastric, intestinal, rectal, vaginal, or urethral routes.

16. The method of claim 9 wherein the HIV antigen is an HIV lysate.
10
17. The method of claim 9 wherein the HIV antigen is a gp120 glycoprotein.

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FIG.1
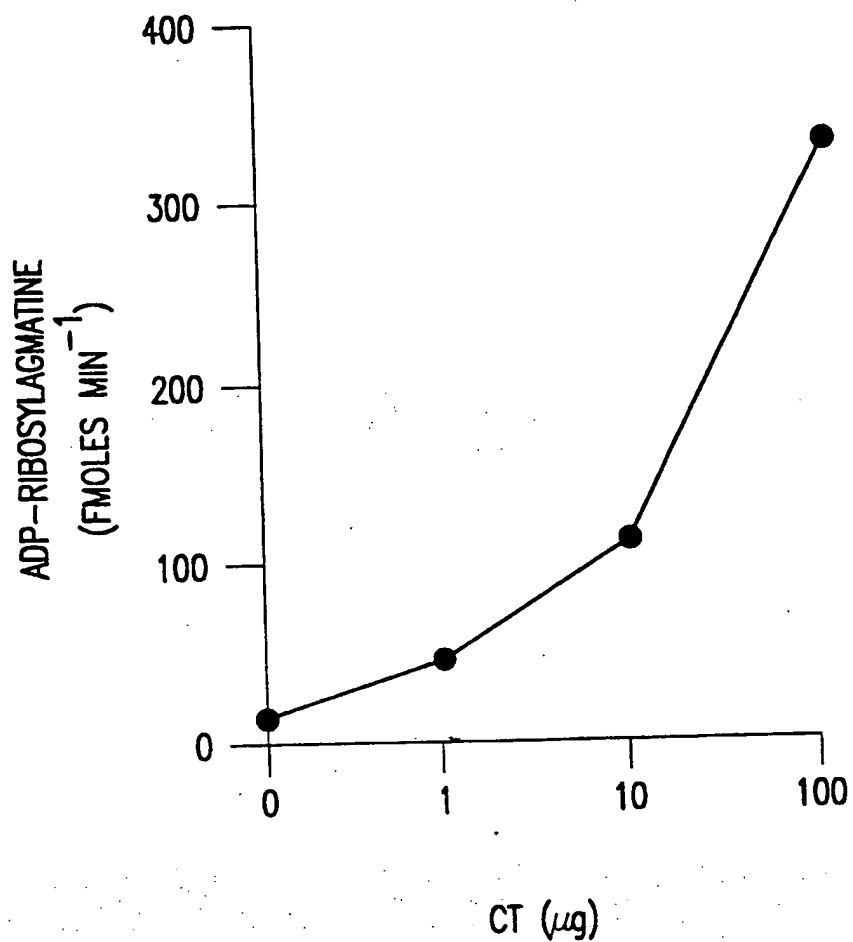
FIG.2
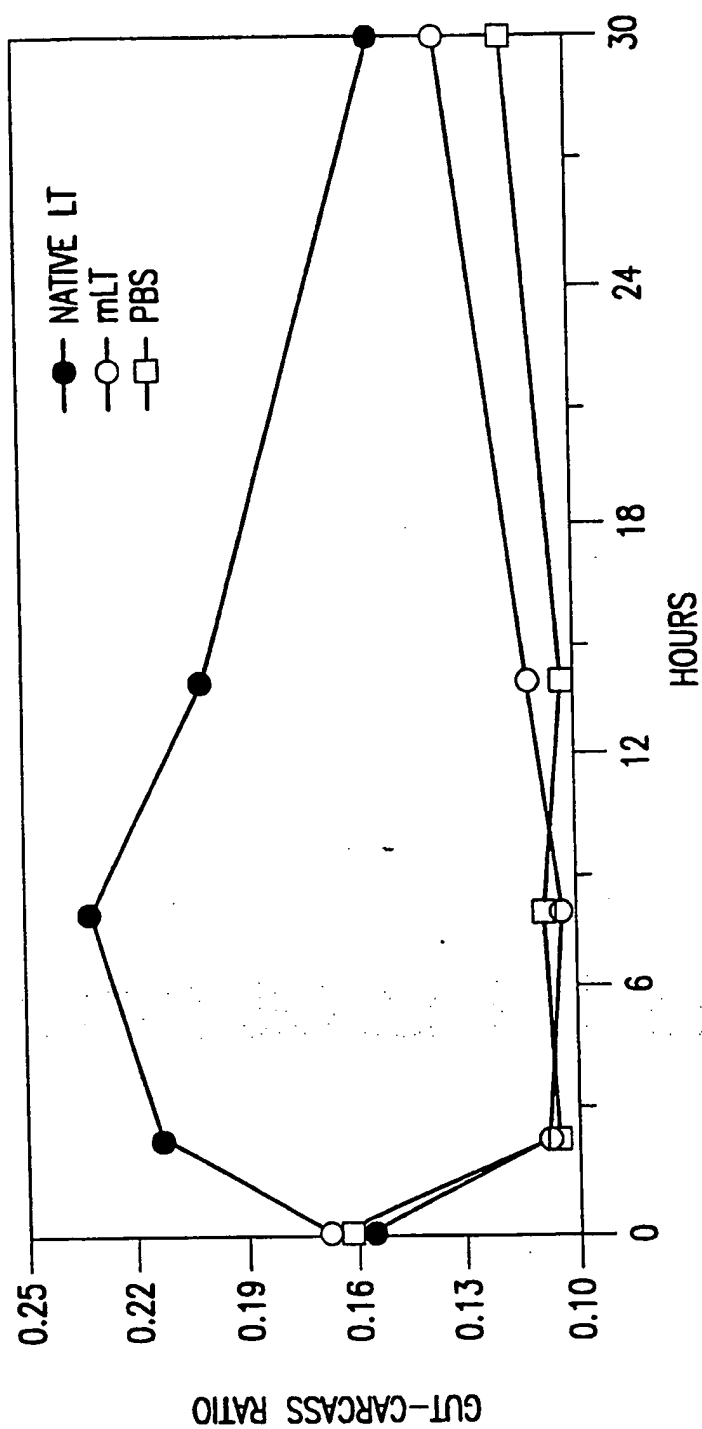
FIG.3

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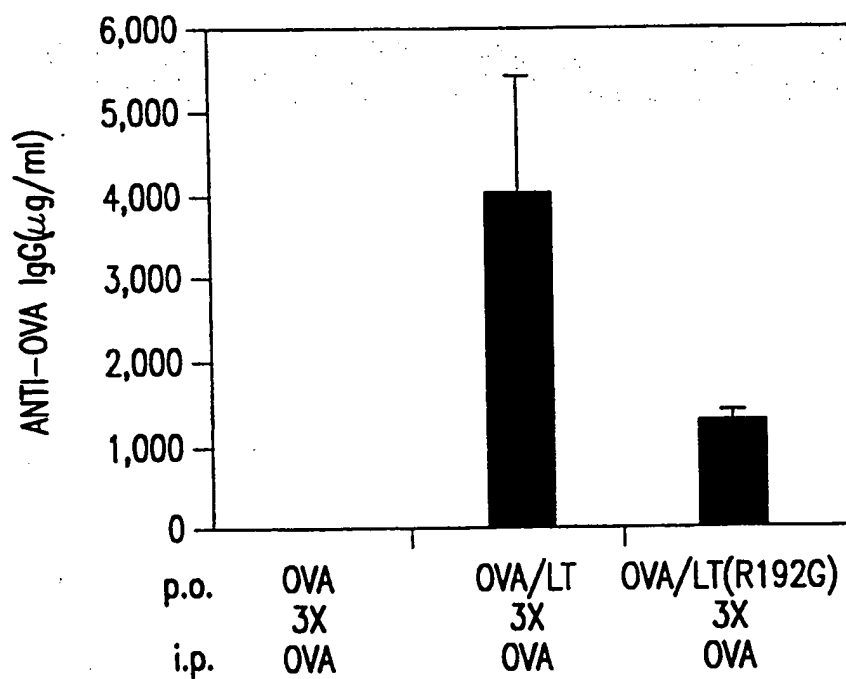
FIG.4A
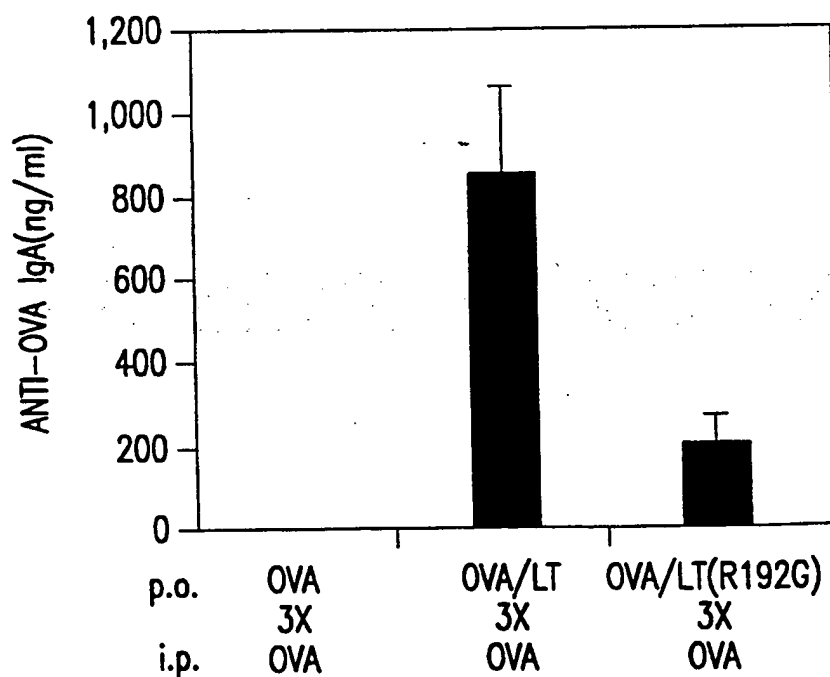
FIG.4B
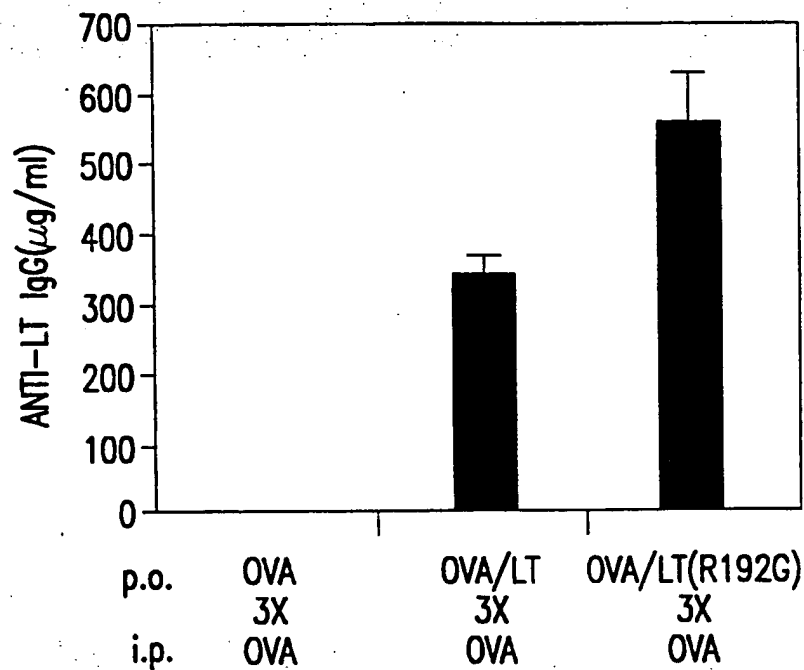
FIG.5A
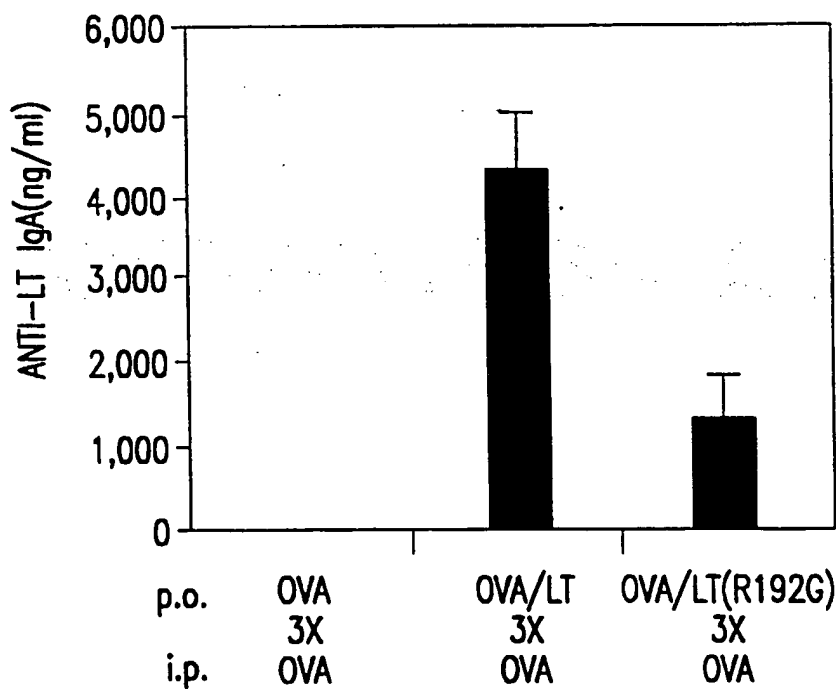
FIG.5B
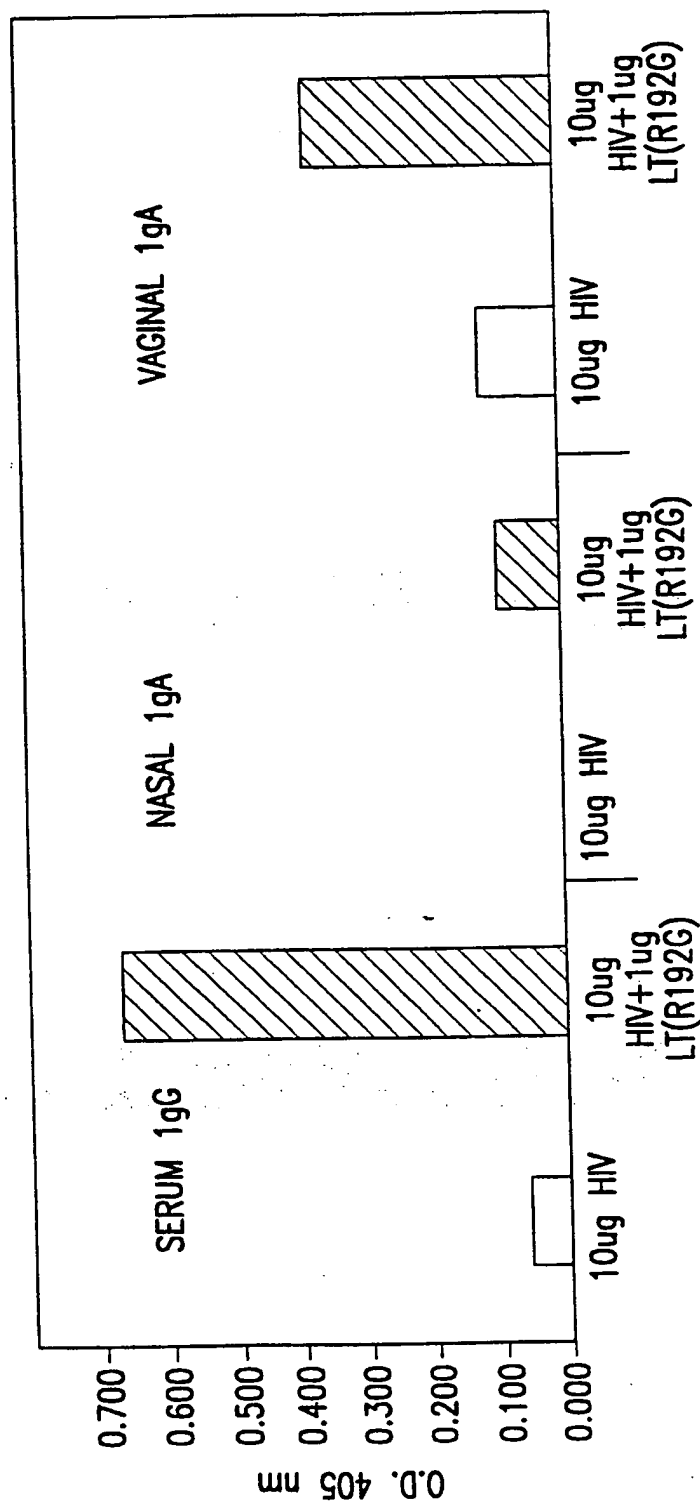
FIG.6
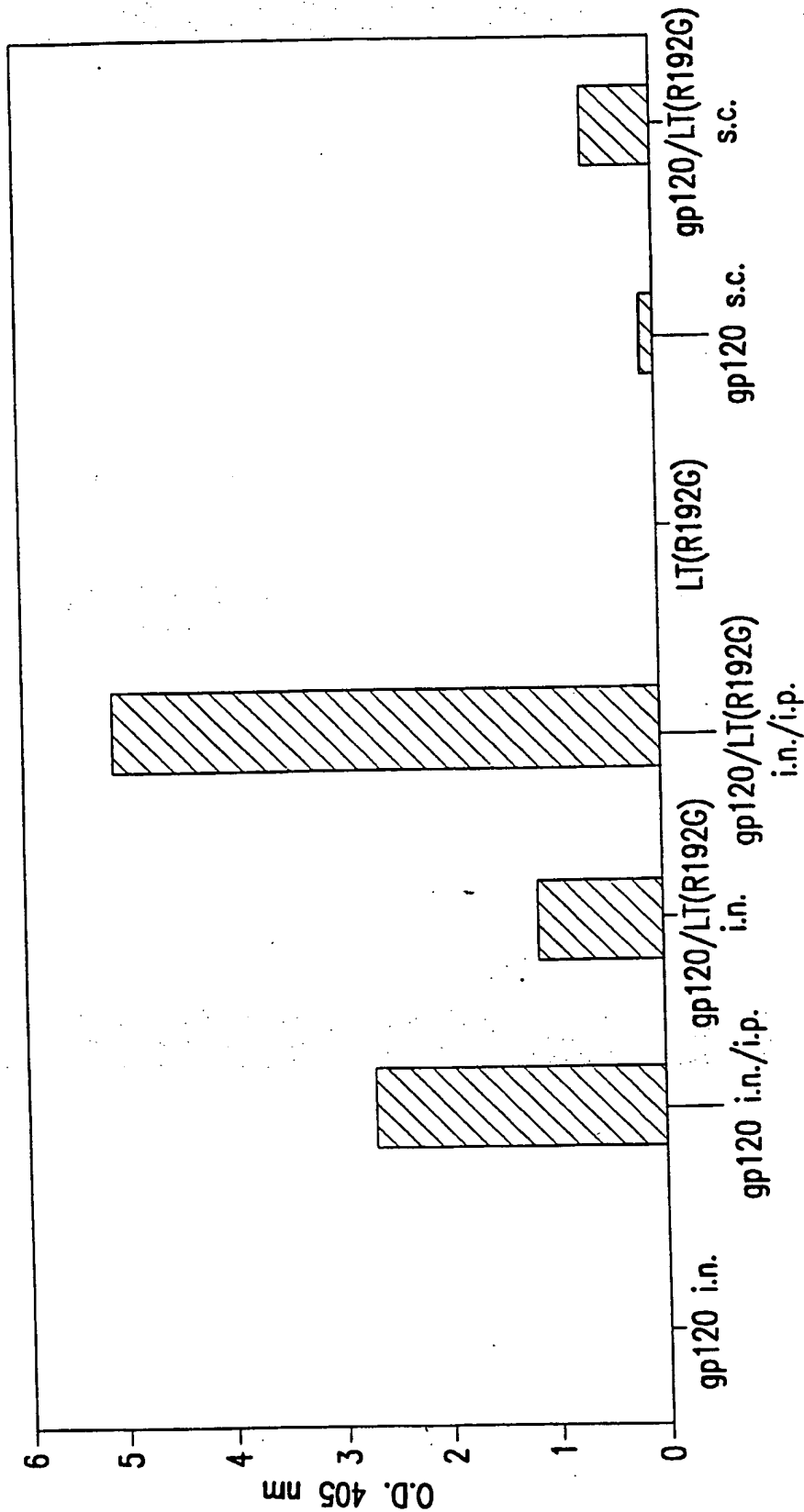
FIG.7
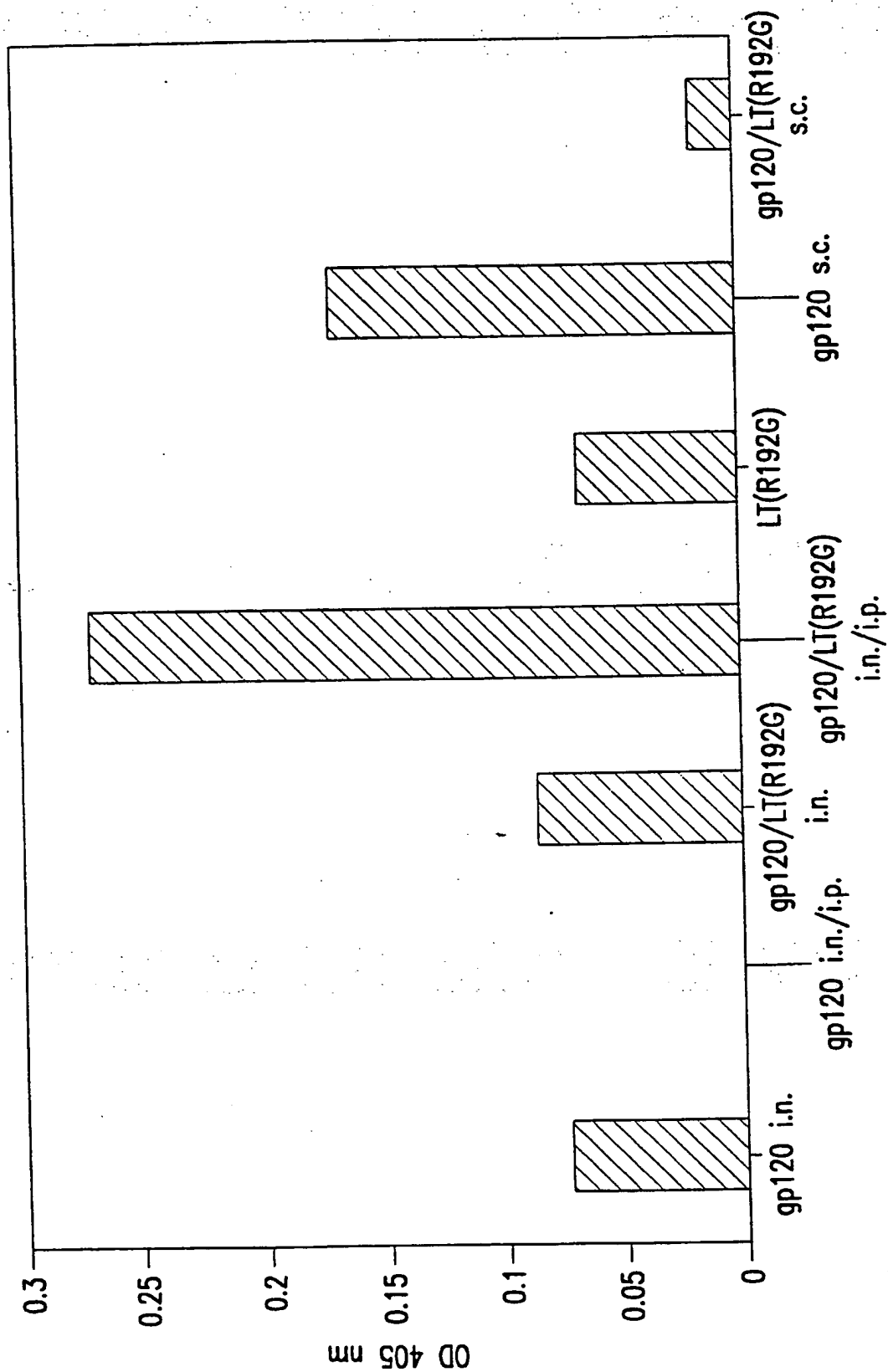
FIG.8

9/9

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : A61K 39/21, 39/108, 39/106; C12N 15/64, 15/00

US CL : 424/208.1, 241.1, 261.1; 435/91.4, 172.1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/208.1, 241.1, 261.1; 435/91.4, 172.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, CAPLUS, MEDLINE, SCISEARCH, BIOSIS, EMBASE, WPIDS, DIALOG

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 93/13202 A1 (BIOCINE SCALVO SPA) 08 July 1993. See especially pages 7, 10, 14-17.	1-17
Y	LOOSMORE, S.M. et al. Engineering of Genetically Detoxified Pertussis Toxin Analogs for Development of a Recombinant Whooping Cough Vaccine. Infection and Immunity. November 1990, Vol. 58, No. 11, pages 3653-3662. See especially page 3653.	1-11
Y	WILLIAMS, D.P. et al. Cellular Processing of the Interleukin-2 Fusion Toxin DAB_{486}-IL-2 and Efficient Delivery of Diphtheria Fragment A to the Cytosol of Target Cells Requires $Arg1^{94}$. Journal of Biological Chemistry. 25 November 1990, Vol. 265, No. 33, pages 20673-20677. See especially page 20674-20675.	1-17

[X] Further documents are listed in the continuation of Box C.   [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance		
"B"	earlier document published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
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Date of the actual completion of the international search	Date of mailing of the international search report
02 MARCH 1998	0 2 JUN 1998

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C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,182,109 A (TAMURA et al.) 26 January 1993. See especially columns 1 and 2	1-17
Y	BACKSTROM, M. et al. Insertion of a HIV-1-neutralizing Epitope in a Surface-Exposed Internal Region of the Cholera Toxin B-subunit. Gene. 1994, Vol. 149, pages 211-217. See especially Abstract	1-17
Y	HOLMGREN, J. et al. Cholera Toxin and Cholera-B subunit as Oral Mucosal Adjuvant and Antigen Vector Systems. Vaccine. 1993, Vol. 11, No. 12, pages 1179-1184. See especially page 1182, and 1183.	1-17